REMARKS

A. Preliminary Remarks

Claims 1-14 and 16 are pending in the application. Claim 1 is amended and claims 6-9 and 14 are canceled by amendment herein. Accordingly, following entry of the present amendment, claims 1-5, 10-13 and 16 will be pending in the application. The amendment to claim 1 is supported throughout the application as filed, including at page 4, lines 22-23 and in Examples 2-4 at page 6, line 31 to page 9, line 27. The amendment does not introduce new matter.

Applicants acknowledge that the Examiner has withdrawn the objection to the specification as amended (new matter), the rejection of claims 1-5 under 35 U.S.C. § 112, first paragraph, for lack of written description (new matter), the rejection of claims 1-14 and 16 under 35 U.S.C. § 112, first paragraph, for lack of enablement, and the rejection of claims 1-14 and 16 under 35 U.S.C. § 103(a) over Advani (1998) in view of Carroll. The sole issue with respect to the patentability of the pending claims is the rejection of claims 1-14 and 16 under 35 U.S.C. § 103(a) over Advani (1997) (abstract) in view of Carroll.

B. The rejection of claims 1-14 and 16 as obvious over Advani (1997) in view of Carroll should be withdrawn

The Examiner maintained the rejection of claims under 35 U.S.C. § 103(a) over Advani (1997) in view of Carroll. In supporting remarks, the Examiner initially acknowledged that Advani (1997) "does not explicitly teach that the attenuated HSV virus [R7020] could be used to treat a non-CNS tumor in vivo." Office Action at page 3. The Examiner then asserted that "Carroll teaches treatment of non-CNS tumor using an attenuated HSV (hrR3)." Id. The Examiner concluded that "[o]ne of ordinary skill in the art would have been motivated to modify the method of Advani to treat a non-CNS cancer because Carroll teaches that attenuated HSVs can be used to treat non-CNS-type tumors." Office Action at page 4. In response, Applicants disagree with the Examiner's position and traverse the rejection.

As amended herein, the rejected claims have either been canceled or drawn to methods of reducing non-CNS tumors, which the Examiner has acknowledged is not taught by the primary reference, Advani (1997). Even with respect to the gliomal (CNS) xenograft disclosed in Advani (1997), moreover, the reference does not show that R7020, or R3616, would be useful in treating such xenografts. Advani (1997) states that "[t]umors were harvested 3, 5, 7, and 14 days after viral injection." The graphs showing viral yield as a function of time post-infection show that the yields of each of R3616 and R7020 drop to zero prior to day 14, a day on which xenograft tumors were still being harvested from mice. Thus, all of the viral therapeutic is apparently lost from the mice prior to eradication of the xenograft tumor. Advani (1997) does not disclose or suggest that R7020 (or any other HSV) would be effective in treating a CNS tumor or a non-CNS tumor.

One of skill in the art would understand that a method of treating any tumor would need to provide a safe therapeutic in addition to providing an effective therapeutic. The selective destruction of tumor cells requires that the therapeutic, such as an attenuated HSV, be capable of destroying tumor cells while not destroying healthy cells. Advani (1997) says nothing about the effect that HSV R7020 might have on healthy cells *in vivo*. Advani (1997) disclosed the effect of ionizing radiation on viral replication in gliomal xenografts in mice. As such, the reference disclosed that tumors were harvested at various time points post-infection and all subsequent work was done *in vitro* on the isolated xenograft tumors. Nothing is provided about the condition of the mice or any of the healthy tissues of those mice, and that is unsurprising in view of the focus of the work on viral replication in the xenografts.

The disclosure in Advani (1997) that attenuated HSV replicates in gliomal cells is also unremarkable. HSV was known in the art as a neurotrophic virus and, hence, all wild-type and moderately attenuated HSV would be expected to replicate in CNS cells, including glial cells. Accordingly, Advani (1997) neither discloses nor suggests a method of treating a non-CNS tumor comprising administering R7020, or any other HSV. The Examiner, however, relies on Carroll rather than Advani (1997) for the suggestion to modify the method of Advani (1997) to a method involving a non-CNS tumor. As noted above, Advani (1997) does not disclose or suggest any method of treating a tumor, CNS or non-

CNS, because it does not disclose or suggest that the attenuated HSV are effective (i.e., destroy tumors) or safe (i.e., do not destroy healthy tissues). Even if modification of the method of Advani (1997) to a non-CNS tumor context would yield a method of treating a non-CNS tumor, moreover, Carroll does not fairly suggest such a modification.

The Examiner and Applicants appear to agree that Advani (1997) disclosed the implantation of gliomal xenografts into the hindlimbs of mice and the injection of attenuated HSV into those xenografts. The Examiner and Applicants disagree, however, that "Carroll teaches a method for treating colon carcinoma liver metastasis by administering an attenuated HSV directly to the tumor (e.g., see abstract)." Office Action at page 3. The Carroll abstract disclosed that colon carcinoma cells were injected intrasplenically, with a subsequent intrasplenic injection of HSV hrR3. One of ordinary skill would understand that intrasplenic injection is an indirect route for delivery of compositions, via the portal vein, to the liver and beyond. See, e.g., Nichols et al., Archives of Pharmacol., 335:344-350 (1987) (abstract only attached as Appendix A). Consistent with that accepted understanding of intrasplenic administration, Carroll does not even mention the presence or absence of any tumor in the spleen; rather, Carroll discloses liver metastases. Thus, the colon carcinoma cells and the attenuated HSV were both delivered indirectly to the liver. Importantly, the attenuated HSV was not administered directly to any tumor, unlike the direct delivery of Advani (1997). Thus, the method of Advani (1997) could not be modified by simple extension to non-CNS tumors because Advani (1997) taught direct injection into tumors and Carroll taught indirect administration.

In addition, Applicants submit that the Examiner's generalization of Carroll as teaching the administration of an attenuated HSV is improper. Carroll discloses a particular attenuated HSV, i.e., HSV hrR3. The HSV hrR3 is an HSV having an insertionally inactivated U_L39 that is therefore incapable of expressing ICP6. Consequently, HSV hrR3 produces no active ribonucleotide reductase, an enzyme needed for viral replication. Not any attenuated HSV would satisfy this criterion and, therefore, Carroll cannot be generalized as disclosing an attenuated HSV.

As noted in Carroll itself, HSV hrR3 was chosen as the attenuated HSV because colon carcinoma cells produce large quantities of ribonucleotide reductase while healthy liver cells express little or no ribonucleotide reductase. Thus, HSV hrR3 was expected to be useful because it could replicate in, and ultimately kill, colon carcinoma cells by using cellular ribonucleotide reductase, but it would not have been expected to replicate in (nor kill) healthy liver cells because of a lack of ribonucleotide reductase. Given the absence of viral ribonucleotide reductase, Carroll found HSV hrR3 sufficiently attenuated to be worth exploration in a non-CNS tumor model. Consistent with this observation is the statement in the first publication characterizing HSV hrR3 that "[a] fine balance must be achieved between optimizing the efficacy of tumor cell killing, minimizing spread of the virus in non-tumor tissue, and safety options." Mineta et al., Cancer Res. 54:3963-3966 (1994), at page 3966 (Appendix A).

The inactivation of *U_L39* in Carroll's HSV hrR3 can be analogized to the deletion of both copies of *$\gamma_1 34.5$* in Advani (1997)'s HSV R3616 insofar as each virus is known to completely lack an important gene product that results in an attenuation of the virus. In contrast, the genotype of HSV R7020 (Advani (1997)) is markedly different from the genotype of Carroll's HSV hrR3 (or HSV R3616).

HSV R7020 is an HSV-1/HSV-2 intertypic recombinant that contains a large deletion of the joint region, including *U_L56* and one copy of $\gamma_1$34.5, a duplication of *U_L5* and *U_L6*, insertion of HSV-2 gJ, gG, and PK, and deletion of *U_L24*. The loss of *U_L24* and *U_L56* means that HSV R7020 has lost non-essential genes encoding a possible membrane-associated protein and a virion-associated protein of unknown function, respectively. Roizman et al., Proc. Natl. Acad. Sci. (USA) 93:11307-11312 (1996) (Appendix A). Unlike the HSV hrR3 of Carroll, however, HSV R7020 does not contain a mutated *U_L39*. (Also, unlike HSV R3616, HSV R7020 does not contain a deletion of both copies of the *$\gamma_1 34.5$* gene.) Thus, HSV R7020 was recognized in the art as distinguishably less attenuated than the HSV hrR3 of Carroll.

The reduced attenuation of HSV R7020 relative to HSV hrR3 (Carroll) means that one of skill in the art would not have expected the results obtained with HSV R7020 in

the CNS context to be extendable to the use of HSV R7020 in the non-CNS context, regardless of any teaching by Carroll concerning the results obtained with the more highly attenuated HSV hrR3 in a non-CNS context. As established above, Advani (1997) does not disclose or suggest any effect of HSV R7020 on healthy tissue (CNS or non-CNS). Carroll did not even try the less-attenuated HSV R7020 and, thus, could not disclose or suggest that such a virus would be useful in treating (i.e., selectively killing) non-CNS tumor cells. Consequently, the combined teachings of Advani (1997) and Carroll do not provide a disclosure or suggestion that a virus according to the pending claims, such as HSV R7020, would be useful in a method of treating a non-CNS tumor.

The requirement not only for efficacy in killing tumor cells but safety in not killing healthy cells was underscored by a publication of the oncolytic use of the more highly attenuated HSV R3616. The authors of that publication expressly stated that "[s]tudies are now needed to explore the effects of viruses engineered to contain multiple mutations, which may further reduce neurovirulence and the possibility of wild type-reversion." See Markert et al., Neurosurg. 32:597-603 (1993) at page 602 (Appendix A). Even the authors of Carroll remained concerned about the relatively highly attenuated HSV hrR3 in stating that "we anticipate that modifications to address three issues will enhance the safety and efficacy of HSV1-based oncolytic therapy." Yoon et al., FASEB J. 14:301-311 (2000) at page 310 (Appendix A). The first issue was the possible reversion of HSV hrR3 to wild-type HSV by expulsion of the *lacZ* gene used to insertionally inactivate U_L39 in that virus. The second issue was the possible difficulty in killing G_0 cancer cells with HSV hrR3 and the third issue was the possible killing of dividing, but non-cancerous, cells by HSV hrR3. In view of these concerns in the art, which have persisted, one of skill would have looked to use more highly attenuated HSV in methods of treating non-CNS tumors, and would certainly not have expected less attenuated HSV to be useful in achieving the efficacy and safety needed to treat non-CNS tumors.

For the foregoing reasons, Applicants submit that Advani (1997) in combination with Carroll fails to disclose or suggest each element of any one of the claims pending upon entry of the present amendment. In particular, neither Advani (1997) nor Carroll, taken alone or in combination, disclose or suggest the use of an HSV having one

copy of the $\gamma_1 34.5$ gene expressing an active gene product in a method of treating a non-CNS tumor because the cited art does not disclose or suggest the efficacy of using HSV R7020 (or any like virus defined by the claims) to treat any tumor and does not disclose or suggest the safety required in any such treatment. Consequently, Advani (1997) in combination with Carroll cannot disclose or suggest the claimed methods of treating non-CNS tumors. Because the burden is on the Examiner to establish that each element of each rejected claim is disclosed or suggested in the cited references and that has not been achieved, a *prima facie* basis for rejecting any of the claims as obvious under 35 U.S.C. § 103(a) has not been established.

In addition to not identifying each claim element in the combined references, there is no proper motivation to combine Advani (1997) and Carroll. The significant differences in the relatively unattenuated HSV R7020 of Advani (1997) and the relatively highly attenuated HSV hrR3 of Carroll means that one cannot find a motive to extend the use of the less safe R7020 from the CNS tumor environment to the non-CNS tumor environment simply because Carroll purports to disclose the use of a different, and much safer, HSV in the non-CNS environment. For this reason as well, a *prima facie* basis for rejecting any of the claims as obvious has not been established.

Finally, because each of the claim elements has not been disclosed or suggested in Advani (1997) and Carroll, considered as a whole, there can be no reasonable expectation of successfully arriving at the claimed subject matter.

Applicants respectfully submit that none of the three criteria necessary to establish a *prima facie* basis for rejecting any of the instant claims as obvious under 35 U.S.C. § 103(a) over Advani (1997) in view of Carroll has been satisfied. Consequently, the rejection should be withdrawn.

C. Conclusion

For all of the foregoing reasons, Applicants submit that all outstanding rejections and objections have been overcome and claims 1-5, 10-13, and 16 are in condition for allowance. An early notice thereof is respectfully solicited.

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Respectfully submitted,

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Appendix A

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Summary In order to determine whether or not glucagon released from the pancreas might have local vascular effects, the actions upon regional haemodynamics in the anaesthetised rat of two doses of glucagon (2 and 10 μg kg^{-1} min^{-1}) infused intrasplenically (and thus into the portal vein) were compared with those of a single dose (2 μg kg^{-1} min^{-1}) infused i. v. Infusion of glucagon i. v. produced a significantly increased heart rate (by 6%) and cardiac output (by 23%) in the experimental animals compared to those receiving saline by the same route. Total peripheral resistance fell by 24%. A greater proportion of the cardiac output passed to the coronary and renal vascular beds and blood flow was increased in the spleen, testes, pectoral skeletal muscle, stomach and small intestine as well as the heart and kidneys.

The lower dose infused intrasplenically had no significant effect on cardiac output or total peripheral resistance but significantly increased the proportion of cardiac output passing both to the stomach and the small intestine such that the percentage of cardiac output flowing through the portal vein increased from 19.1 ± 1.1% to 23.8 ± 1.7%.

Intrasplenic infusion of 10 μg kg^{-1} min^{-1} significantly increased cardiac output (by 29%) but reduced total peripheral resistance by 37%. Greater fractions of the cardiac output were received by the spleen, small intestine and epididymides. Blood flow was increased in these organs and the skin, kidneys, stomach, large intestine and the mesentery.

It is concluded that pharmacologically effective amounts of glucagon only passed into the systemic circulation with the higher dose infused intrasplenically. Thus the redistribution of cardiac output in favour of the splanchnic bed with the lower dose of glucagon infused into the portal region is most likely the result of local mechanisms rather than a direct effect of the hormone on the inflow vasculature resulting from recirculation.

Key words Glucagon - Rat regional haemodynamics - Gastrointestinal blood flow - Intrasplenic administration

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Experimental Therapeutics, Molecular Targets and Chemical Biology

Oncolytic Herpes Simplex Virus Vector Therapy of Breast Cancer in C3(1)/SV40 T-antigen Transgenic Mice

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Abstract

Oncolytic herpes simplex virus vectors are a promising strategy for cancer therapy, as direct cytotoxic agents, inducers of antitumor immune responses, and as expressers of anticancer genes. Progress is dependent upon representative preclinical models to evaluate therapy. In this study, two families of oncolytic herpes simplex virus vectors (G207 and NV1020 series) that have been in clinical trials were examined for the treatment of breast cancer, using the C3(1)/T-Ag transgenic mouse model. Female mice spontaneously develop mammary carcinomas, and the C3(1)/T-Ag–derived tumor cell line M6c forms implantable tumors. Both *in vitro* and *in vivo*, G47Δ, derived from G207 by deletion of ICP47 and the US11 promoter, was more efficacious than G207. Whereas NV1023, derived from NV1020 by deletion of ICP47 and insertion of LacZ, was as cytotoxic to M6c cells *in vitro* as G47Δ, it did not inhibit the growth of s.c. M6c tumors but did extend the survival of intracerebral tumor bearing mice. In contrast, NV1042, NV1023 expressing interleukin 12, inhibited s.c. M6c tumor growth to a similar extent as G47Δ, but was less effective than NV1023 in intracerebral tumors. In the spontaneously arising mammary tumor model, when only the first arising tumor per

mouse was treated, G47Δ inhibited the growth of a subset of tumors, and when all tumors were treated, G47Δ significantly delayed tumor progression. When the first mammary tumor was treated and the remaining mammary glands removed, NV1042 was more efficacious than G47Δ at inhibiting the growth and progression of injected tumors.

Key Words: virotherapy • herpes simplex virus • transgenic mouse • mammary adenocarcinoma • brain metastases

Introduction

Breast cancer incidence has continued to increase in the United States, albeit at diminished rates (1). Encouragingly, the mortality rate has been decreasing since 1990 (2). Both these trends are thought to partially reflect increased mammography use. Unfortunately, the latest 5-year survival rate for metastatic disease is only 20.4%, compared with 97.5% for localized disease (2). In about 15% to 30% of patients, the primary cancer will metastasize to the brain (3), with this percentage increasing over the last few decades, related in part to longer survival due to increased efficacy of current treatments of peripheral disease (4). Surgery is the primary treatment for localized tumors, often with radiation, followed by adjuvant chemotherapy and hormonal therapy, depending upon receptor status (5), and more recently biological therapies that target the epidermal growth factor receptor family and downstream signaling (6). Metastasis to the brain frequently results in severe and debilitating neurologic complications, which have a large impact on a patient's quality of life. Therapy for metastatic disease is often only palliative (5) and has minimal effect on survival in patients with brain metastases (7).

Oncolytic viruses are promising therapeutic agents for cancer. They are inherently cytotoxic to tumor cells and conditionally replicative so that spread of the vector is confined to the tumor (8). Major advantages of such vectors are *in situ* amplification and spread within the tumor, ability to transfer therapeutic transgenes to the tumor, and induction of antitumor immune responses. Of course, equally important for clinical translation is that such vectors have minimal toxicity to normal tissue. Herpes simplex virus (HSV) has many properties that make it an attractive cancer therapeutic agent and it has served as a prototypic oncolytic virus (9). A variety of oncolytic HSV vectors have been developed, with three among these, G207, 1716, and NV1020, safely completing phase 1 clinical trials (9).

In this paper, we examine the efficacy of oncolytic HSV vectors from the G207 and NV1020 series. G207 is a multigene mutant of HSV-1 that contains deletions of both copies of the *γ34.5* gene, the major viral determinant of neurovirulence (10) and antagonist of activated double-stranded RNA-dependent protein kinase R (11), and an *Escherichia coli LacZ* insertion that inactivates the *ICP6* gene (*UL39*), encoding the large subunit of ribonucleotide reductase, a key enzyme in nucleotide metabolism and viral DNA synthesis in nondividing cells (12, 13). G207 is efficacious in the treatment of multiple human tumors in athymic mice and mouse tumors in syngeneic animals, yet is nonpathogenic to HSV-sensitive mice and nonhuman primates (14). In addition to its oncolytic activities, G207 infection of tumor cells in

immunocompetent mice induces a systemic and specific antitumor immune response (15–18). Whereas the mutations in G207 confer significant safety attributes (13), they also attenuate viral growth.

To enhance the antitumor activities of G207, we generated G47Δ, which contains an additional deletion of the nonessential *α47* gene (ICP47; ref. 19). Because of the overlapping transcripts encoding ICP47 and US11, this deletion places the late *US11* gene under control of the immediate-early *α47* promoter, which results in an enhancement of growth of *γ34.5*$^-$ mutants and broadens the range of susceptible tumor cells by precluding the shutoff of host protein synthesis (20). ICP47 binds to the transporter associated with antigen presentation (TAP) and blocks peptide loading of MHC class I molecules (21). Its deletion, therefore, leads to increased MHC class I presentation on infected cells, enhanced stimulation of lymphocytes, and decreased NK cytolysis (19, 22), which should augment the induction of an immune response. Unfortunately, ICP47 is unable to inhibit rodent TAP, making mice an unsuitable model to examine the impact of ICP47 *in vivo* (23).

NV1020 (previously called R7020) is a HSV-1/HSV-2 intertypic recombinant that was developed and unsuccessfully tested in humans as a herpes vaccine (24, 25). It contains a large deletion of the joint region, including *UL56* and one copy of *γ34.5*, a duplication of *UL5* and *UL6*, insertion of HSV-2 *gJ*, *gG*, and *PK*, and deletion of *UL24* (24, 26). Because it is not attenuated for neuroinvasiveness, it has been tested against non-CNS tumors (27), including colon cancer metastatic to the liver in mice and humans (28, 29). NV1023 is derived from NV1020 by repairing the *thymidine kinase/UL24* region, and insertion of *E. coli LacZ* into the *ICP47* region, deleting *ICP47* and *US11* (30). This vector was used as the backbone to generate NV1042, which expresses murine interleukin (IL)-12 (30). IL-12 expression has previously been shown to enhance the efficacy of oncolytic HSV therapy and augment the antitumor immune response generated (30–32).

Representative animal models are central to the development and testing of novel therapeutic strategies. As a model for breast cancer, we have used the C3(1)/T-Ag transgenic mouse, which spontaneously develops mammary adenocarcinoma in female mice due to the expression of SV40 large T- and small t-Ag driven by the rat prostatic steroid binding protein C3(1) enhancer/promoter (33, 34). Transgene expression is not estrogen responsive, although estrogen promotes tumorigenesis, so tumor development is not pregnancy or hormone dependent (35). These transgenic mice typically develop atypical hyperplasia in the ducts at about 2 months of age, followed by high-grade mammary intraepithelial neoplasia at 3 months and adenocarcinomas beginning at 4 months (34). Breast cancer cell lines have been established from these mice that form tumors after implantation into heterozygous C3(1)/T-Ag mice (36), including intracerebral tumors that are a model for metastatic disease to the brain. Here, we compared the efficacy of G47Δ and NV1023 in implanted s.c. and intracerebral breast cancer tumors, and determined the impact of IL-12 expression on oncolytic HSV therapy of both implanted (s.c. and intracerebral) and spontaneous mammary tumors. To our knowledge this is the first described treatment of spontaneously arising tumors by oncolytic viruses.

Materials and Methods

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Cells and Viruses. M6, M6c, and Pr14-2 tumor cells (obtained from Dr. J. Green, National Cancer Institute, Bethesda, MD) isolated from C3(1)/T-Ag tumors (36, 37) and Vero (African green monkey kidney; obtained from D. Knipe, Harvard Medical School, Boston, MA) cells were cultured in DMEM with glucose (4.5 g/l; Mediatech, Inc., Herndon, VA) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT) at 37.5°C in 5% CO_2.

G207 and G47Δ were constructed as described (13, 19). NV1023, derived from NV1020 (R7020), contains an insertion of *LacZ* into the *ICP47* locus, deleting *ICP47*, *US11*, and *US10* (30). NV1042 is NV1023 with an insertion of murine IL-12 cDNA (p35 and p40 as a single polypeptide separated by elastin motifs) expressed from a hybrid *α4-TK* promoter (30). Purified virus stocks in Dulbecco's PBS/10% glycerin were provided by MediGene, Inc. (San Diego, CA). Viruses were titered by plaque assay on Vero.

***In vitro* Cytotoxicity.** Cells were seeded in 6-well plates at 1×10^5 cells per well. After a 24-hour incubation at 37.5°C, cells were infected with virus at a multiplicity of infection (MOI) of 0.1, whereas controls were Mock infected. The infected cells were incubated at 37.5°C until counting, when they were washed twice with PBS, trypsinized and counted with a Coulter counter (Beckman Coulter, Fullerton, CA).

Animal Studies. Six-week-old female C3(1)/T-Ag heterozygous transgenic mice were purchased from the National Cancer Institute (Frederick, MD) and caged in groups of five or less. Mice were genotyped using the online Jackson Laboratory genotype protocol with primers for T-Ag (5'-CAGAGCAGAATTGTGGAGTGG-3' and 5'-ACAAACCACAACTAGAATGCAGTG-3'). Because M6c cells still express T-antigen, they only form tumors in C3(1)/T-Ag transgenic mice. For injections and surgical procedures, each mouse was anesthetized with an i.p. injection of 0.20 to 0.25 mL solution consisting of 86% saline, 9% sodium pentobarbital, and 5% ethyl alcohol. Animal procedures were approved by the Massachusetts General Hospital Animal Care and Use Committee. All animal studies were blinded.

S.c. Tumor Model. M6c cells (1×10^6 in 50 μL of serum-free DMEM) were injected s.c. into the right flank of 6-week-old female heterozygous C3(1)/T-Ag transgenic mice. When s.c. tumors reached approximately 6 mm in maximal diameter, 20 μL of virus (2×10^7 plaque-forming units, pfu) or vehicle (Mock) was inoculated into the tumor (day 0), followed by repeat injections either on days 6 and 11 (G207) or on days 3, 7, and 10 (G47Δ, NV1023, and NV1042). Tumor size was measured by external caliper twice a week and the tumor volume calculated (length x width x height). If animals seemed moribund or the diameter of their tumors exceeded 21 mm, they were sacrificed and this was recorded as the date of death for survival studies.

Intracerebral Tumor Model. Intracerebral tumors were generated by injecting 2×10^5 M6c cells in 4 μL of serum-free DMEM stereotactically into the right frontal lobe of female heterozygous C3(1)/T-Ag mice (38). After 10 days, 4 μL of virus (2×10^6 pfu) or vehicle (Mock) was inoculated stereotactically at

the same coordinates, and survival was monitored. Survival was statistically evaluated by Kaplan-Meier analysis and log-rank test (StatView).

Spontaneously Arising Mammary Tumors. Female heterozygous C3(1)/T-Ag transgenic mice spontaneously develop mammary tumors that are palpable from about 4 months of age. Mice with palpable mammary tumors were randomly divided into two groups based on the order of tumor development. We used three treatment paradigms: (a) Only the first mammary tumor to arise was injected with G47Δ (1×10^7 pfu/20 μL) or Mock (10% glycerol in PBS) weekly until the animals were sacrificed due to overall tumor burden; (b) All tumors as they arose were injected with G47Δ (1×10^7 pfu/20 μL) or Mock (10% glycerol in PBS) weekly until the animals were sacrificed due to overall tumor burden; and (c) The first mammary tumor to arise was injected with G47Δ or NV1042 (2×10^7 pfu/20 μL) or Mock (10% glycerol in PBS) weekly. Following the first treatment (about 3 days later), the remaining nine mammary glands were surgically removed. Animals were followed until sacrifice due to overall tumor burden. Tumor sizes were measured with calipers twice a week and the volume (length x width x height) determined. In all cases, both the treatments and measurements were blinded. Statistical differences in tumor growth were assessed using an unpaired *t* test and time to progression using a log-rank test of Kaplan-Meier estimates (Prism, GraphPad Software, Inc., San Diego, CA). The time to progression is based on the Response Evaluation Criteria in Solid Tumors criteria, where a 30% increase in maximal diameter in a spherical tumor will result in a new volume = $4/3\pi \times (1.3r)^3$ or 2.2 x the original volume, a 120% increase in volume (39). For the rapidly growing spontaneous tumors, the 30% increase in maximal diameter is more appropriate than the standard 20% increase (or 73% increase in volume) used in clinical trials as the determinant of progressive disease for tumor response evaluation (39).

Histology. Mice were sacrificed and perfused with Zamboni's fixative [1.8% paraformaldehyde, 7.5% picric acid, 0.19% EGTA and 2 mmol/L magnesium chloride (pH 7.3)]. Tumor samples were harvested, frozen with dry ice, and cryostat sections of 20-μm thickness were prepared. Sections were fixed in 2% paraformaldehyde/PBS for 10 minutes, washed twice in PBS, incubated with PBS containing 2 mmol/L magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP40 at 4°C for 10 minutes, and then stained with substrate solution [PBS (pH 7.2) containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP40] at 34°C for 4 hours. Sections were washed with PBS/2 mmol/L EDTA and counterstained with H&E before mounting.

Results

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***In vitro* Cytotoxicity.** To assess the susceptibility of murine breast cancer cells to oncolytic HSV vector cytotoxicity and replication before *in vivo* experimentation, monolayers of C3(1)/T-Ag tumor cells were infected with G207, G47Δ, NV1023, and NV1042 at low MOI (Fig. 1*A*). M6 cells were established from a spontaneously arising mammary adenocarcinoma in C3

(1)/T-Ag mice (36), M6c cells from a lung metastasis arising after s.c. implantation of M6 cells (36), and Pr14-2 from a prostate adenocarcinoma arising in a male C3(1)/T-Ag mouse (37). G47Δ was more effective than G207 at killing all three tumor cell lines, with >60% of cells killed within 3 days at a low MOI of 0.05 (Fig. 1*A*). At high MOI (=1), both G207 and G47Δ killed M6 and M6c cells within 2 days (data not shown). NV1023 was similarly cytotoxic as G47Δ toM6c cells but was not effective in the parental M6 cells (Fig. 1*A*).

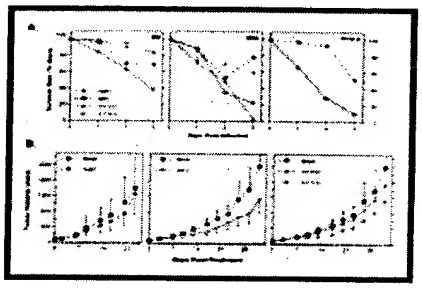

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Figure 1. Oncolytic HSV treatment of C3(1)/T-Ag tumor cells. *A, in vitro* susceptibility of murine C3(1)/T-Ag tumor cells to oncolytic HSV vectors. Monolayers of M6 (*left*) and M6c (*middle*) cells were infected with G207, G47Δ, NV1023, or NV1042 at a MOI = 0.05, and Pr14-2 cells (*right*) with G207, G47Δ at a MOI = 0.1, or Mock, and incubated in DMEM/1% heat-inactivated FCS at 37.5°C for the time indicated. Average from duplicate or triplicate (Pr14-2) wells. *B,* treatment of s.c. M6c tumors. M6c cells were implanted s.c. in the right flank of female C3(1)/T-Ag heterozygous mice. *Left,* when tumors were palpable (7 days after implantation), they were injected intratumorally with G207 (2 x 10^7 pfu/20 μL) or Mock (PBS/10% glycerol) on days 0, 6, and 11 (post-treatment). In a second experiment (*middle* and *right*), palpable tumors (13 days after implantation) were injected intratumorally with G47Δ, NV1023, NV1042 (2 x 10^7 pfu/20 μL) or Mock (PBS/10% glycerol) on days 0, 3, 7, and 10 (post-treatment). *Points,* $n = 7$ (*left*) or $n = 9$ (*middle* and *right*) animals per group; *bars,* SD. Tumors treated with G47Δ and NV1042 were significantly smaller than Mock from 10 days post-treatment ($P < 0.05$, Student's *t* test). There was no significant difference between G207 or NV1023 and Mock.

Treatment of S.c. Tumors. M6c cells were used for the *in vivo* studies because they were more susceptible to oncolytic HSV vector replication *in vitro* than M6 cells, and M6 had highly variable tumor growth rates after s.c. implantation. S.c. M6c tumors were established in female C3(1)/T-Ag heterozygous mice and then injected intratumorally with vector. Similar to what was seen *in vitro*, G207 was unable to inhibit the growth of s.c. M6c tumors (Fig. 1*B, left*) or to extend survival (data not shown). We next examined the efficacy of G47Δ, which significantly inhibited the growth of s.c. M6c tumors ($P < 0.05$, Student's *t* test; Fig. 1*B, middle*). In contrast, NV1023, which replicated well in M6c cells *in vitro*, had no significant effect on M6c s.c. tumor growth (Fig. 1*B, right*). Previous studies from our laboratory have shown that intratumoral expression of IL-12 significantly enhances antitumor efficacy of oncolytic HSV vectors (31). Similarly here, NV1042 was more efficacious than NV1023 and significantly inhibited s.c. tumor growth ($P < 0.05$, Student's *t* test; Fig. 1*B, right*). Both G47Δ and NV1042 significantly extended the survival of treated mice bearing s.c. tumors, with a mean survival of 44 and 43 days respectively, compared with Mock with a mean of 38 days [$P < 0.05$, log-rank (Mantel-Cox) test], whereas NV1023 had no significant effect.

Intracerebral Tumors. As a model for metastatic breast cancer in the brain, we established M6c intracerebral tumors in female C3(1)/T-Ag heterozygous mice. Cells were implanted stereotactically into the striatum (Fig. 2*A*) and within 10 days multifocal tumors developed (Fig. 2*D*). To determine whether G47Δ replication was occurring *in vivo*, intracerebral M6c tumors were treated 10 days post-implantation, animals sacrificed 1, 2, and 4 days after virus injection, and X-gal histochemistry done on sectioned brains to detect infected cells that contain replicating G47Δ. Large numbers of X-gal-positive tumor cells were seen within 24 hours of G47Δ injection (Fig. 2*B*) and at 2 days (Fig. 2*C*) and 4 days (Fig. 2*D* and *E*) post-infection, with virus staining predominantly surrounding areas of tumor necrosis.

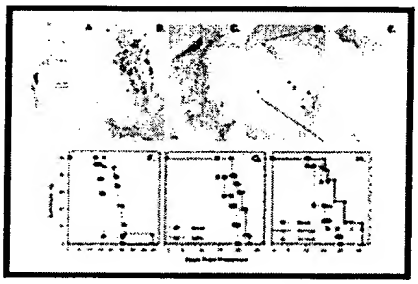

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Figure 2. Treatment of breast cancer metastatic to the brain. (*top*) Stereotactic injection of G47Δ into intracerebral M6c tumors. *A*, cartoon of coronal section through mouse brain illustrating the position of tumor cell and HSV injection. *B*, coronal section through brain of mouse 24 hours after G47Δ (2×10^6 pfu) injection and 14 days after M6c cell implantation. Sections were stained with X-gal to identify cells containing replicating G47Δ (Δ, *blue*), and counterstained with hematoxylin and eosin (tumor deposits, *arrow*). *C*, 48 hours after G47Δ injection. *D*, 4 days after G47Δ injection. *E*, higher magnification of area indicated in *D*. In all cases, the orientation is as in *A*. X-gal-positive cells (*blue*) surrounding tumor deposits can be seen in *B*, *C*, *D*, and *E*. Bar, 0.2 mm in *B*, *C*, and *E*. *Bottom*, oncolytic HSV treatment of M6c intracerebral tumors. *F*, young female C3 (1)/Tag transgenic mice (~7 weeks old) bearing intracerebral M6c tumors were injected at the same coordinates with G47Δ (2×10^6 pfu/4 μL; $n = 9$) or Mock ($n = 12$) and the animals were sacrificed when moribund. G47Δ significantly extended survival [$P = 0.003$; log-rank (Mantel-Cox) test]. Mean survival was increased from 16 days for Mock to 24 days for G47Δ. *G*, M6c cells (4×10^5 cells) were injected stereotactically into aged female C3(1)/Tag transgenic mice (9 months old) and treated 10 days later with G47Δ ($n = 10$) or Mock ($n = 9$). More cells were injected because older mice tend to have lower take rates than young mice. G47Δ significantly extended survival [$P < 0.005$; log-rank (Mantel-Cox) test]. Mean survival was increased from 17 days for Mock to 21 days for G47Δ. *H*, M6c cells (2×10^5) were injected stereotactically into female C3(1)/Tag transgenic mice (~2 months old), 10 days later NV1023 ($n = 8$), NV1042 ($n = 11$; 2×10^6 pfu/4 μL), or Mock (PBS/10% glycerol; $n = 11$) were injected at the same coordinates and the animals were sacrificed when moribund. NV1023 significantly extended survival compared with Mock [$P = 0.004$; log-rank (Mantel-Cox) test], whereas NV1042 was barely significant [$P = 0.05$; log-rank (Mantel-Cox) test]. Mean survival was increased from 14 days for Mock to 20 days for NV1023 and

18 days for NV1042.

We next examined the treatment of established intracerebral M6c tumors with G47Δ in both young mice (~7 weeks of age), a standard model, and in older mice (~9 months of age), more representative of the clinical situation. G47Δ or vehicle (Mock) was stereotactically injected at the same coordinates as the tumor cells and mice followed until moribund, when they were sacrificed. G47Δ significantly extended the survival of tumor-bearing animals at both ages ($P < 0.005$; log-rank [Mantel-Cox] test; Fig. 2*F* and *G*). When we compared the efficacy of NV1023 with NV1042 in the intracerebral tumor model, NV1023 was more effective (Fig. 2*H*), as opposed to the observation in the s.c. tumor model, extending mean survival to 20 days from 14 days for Mock. NV1042 only extended survival to 18 days. In this experiment, the survival of G47Δ treated mice was similar to the NV1042 treated mice (data not shown). All mice that were sacrificed or died had brain tumors.

Spontaneously Arising Mammary Tumors. In our hands, female heterozygous mice develop palpable tumors from about 3 to 5.5 months of age (Fig. 3*A*), although the penetrance is <80%. A mouse with multiple mammary tumors at 6 months of age is illustrated in Fig. 3*B*. G47Δ replication in the tumors could be detected after intratumoral injection from 2 days (Fig. 3*C*) to 7 days post-treatment, with only a few positive cells seen at day 14. Because mammary tumors arise over a period of time and each mouse develops different numbers of tumors, we used three treatment strategies. In the first, mice were randomly divided into two groups (G47Δ and Mock) when the first mammary tumor was palpable. This tumor was injected once a week and tumor size determined twice a week (Fig. 4*A*). There was a high degree of variability in the tumor growth rates in the Mock-treated tumors; however, almost half the tumors exhibited rapid growth to a very large size (Fig. 4*A*, *right*), whereas, none of the G47Δ-treated tumors exhibited such growth and many had somewhat stable disease (Fig. 4*A*, *middle*). The mean tumor volume of the G47Δ-treated tumors was less than the Mock-treated tumors (Fig. 4*A*, *left*). Animals were sacrificed when the tumor burden became too large, with the growth of untreated tumors often leading to the sacrifice of the G47Δ-treated mice. Therefore, there were no apparent treatment effects on survival. There was also no significant difference in the number of mammary tumors arising between the G47Δ (mean = 5.7) and Mock (mean = 5.1) groups.

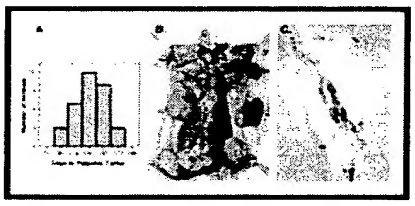

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Figure 3. Spontaneously arising mammary tumors in C3 (1)/T-Ag heterozygous transgenic mice. *A,* time course of tumor development, with the age when the first palpable tumor detected is plotted. *B,* necropsy of mouse (6 months of age) with multiple large mammary tumors (*arrow*). *C,* viral spread in spontaneous mammary tumor after intratumoral inoculation. G47Δ (2×10^7 pfu) was injected into a large mammary tumor (1.4 cm^3) that was removed 2 days later, sectioned, and stained with X-gal histochemistry.

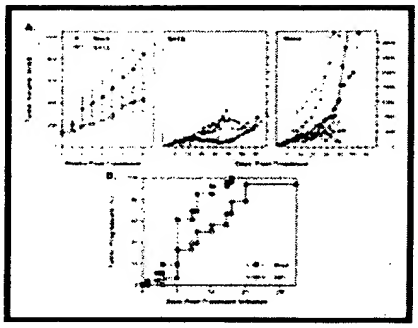

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Figure 4. Treatment of spontaneously arising mammary tumors in C3(1)/T-Ag transgenic mice. *A,* first spontaneous mammary tumor (palpable) was treated with an intratumoral injection of G47Δ (1×10^7 pfu in 20 μL; $n = 8$) or Mock (PBS/10% glycerol; $n = 9$) and weekly thereafter until the mice were sacrificed due to overall tumor burden. Tumor volume = length x width x height. *Left,* whereas the mean tumor volumes of G47Δ treated tumors are smaller than Mock, the difference is not significant ($P \leq 0.07$ at weeks 0.5, 1, 1.5, 3.5, and 4; unpaired t test). However, the slopes of the best-fit lines are significantly different ($P < 0.001$). Because the tumors were measured twice a week, the midweek measurements for different animals could vary by a day; however, they were grouped for purposes of determining the means. *Bars,* standard error of the mean. *Right,* growths of individual treated tumors are plotted. *B,* every spontaneously arising tumor (when it became palpable) was injected with G47Δ ($n = 18$ in six mice) or Mock ($n = 26$ in six mice) weekly and tumor size measured. The time to progression to 2.2 times the original volume at initial treatment (80-420 mm^3) is plotted. G47Δ significantly inhibited tumor progression compared with Mock [$P < 0.01$; log-rank (Mantel-Cox) and Wilcoxon rank tests].

In the second experiment, all tumors were treated when they became palpable, with the mice randomized to treatment groups when the first tumor emerged. These groups also included mice with multiple tumors at the time of first treatment or mice bearing ectopic tumors, those that could not be definitively identified as mammary tumors (i.e., tumors on the neck, flank, and shoulder that could have arisen from sweat glands). In this case, we have treated each tumor as an independent entity and found that G47Δ significantly increased the time to tumor progression (Fig. 4*B*).

In the final treatment paradigm, we treated the first mammary tumor to arise and then surgically removed the other nine breast tissues. In this case, we were able to follow the growth of the injected tumors, for the most part without the confounding effects of subsequent arising tumors. G47Δ did not appreciably inhibit tumor growth, although we were able to follow mean growth longer than for Mock (Fig. 5, *left*). NV1042 did significantly inhibit tumor growth compared with both Mock and G47Δ and delayed tumor progression by over 2.5 times compared with Mock (Fig. 5, *right*). Whereas neither G47Δ nor NV1042 significantly extended overall survival in this experiment, median survival increased from 5.5 weeks for Mock to 8.5 and 9 weeks for G47Δ and NV1042 respectively. Many of these mice were sacrificed due to ectopic tumor burden (usually on the neck or vagina); 62% of Mock, 29% of G47Δ, and 43% of NV1042. All of the Mock-treated tumors progressed to >10 times their initial treatment volume (to ~1,000 mm^3), whereas, only four of seven tumors (57%) in the G47Δ and NV1042 groups progressed to that size. The difference in time to progression to 10 times the initial volume was not quite significant ($P = 0.06$; log-rank test). The effects of G47Δ intratumoral injection on spontaneous mammary tumor growth was not large, but consistent between the different treatment paradigms. The enhanced efficacy of NV1042, consistent with the s.c. tumor study, is supportive of the view that local

IL-12 expression improves oncolytic HSV efficacy.

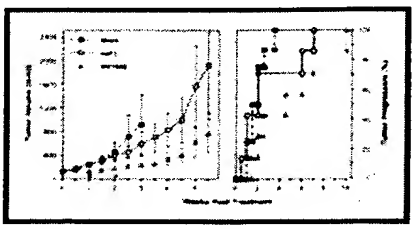

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Figure 5. Oncolytic HSV treatment of single spontaneously arising C3(1)/T-Ag mammary carcinomas. The first spontaneously arising mammary tumor was inoculated with G47Δ or NV1042 (2×10^7 pfu in 20 μL) or Mock (PBS/10% glycerol in 20 μL) weekly. Following the first treatment, the remaining mammary glands were surgically removed. *Left,* tumor growth was followed until the overall tumor burden became too large and the mean tumor volume determined (n = 8 for Mock and n = 7 for NV1042 and G47Δ). NV1042 is more efficacious at inhibiting tumor growth than G47Δ, which was similar to Mock in this experiment, although the difference compared with Mock is only significant at early time points. *, $P < 0.02$ (unpaired t test). *Bars,* standard deviation from the mean. *Right,* time to progression to 2.2 times the initial treatment volume. NV1042 significantly delays tumor progression compared with Mock [$P < 0.02$, log-rank (Mantel-Cox)].

Discussion

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Virotherapy, the use of viruses to treat cancer, has been resurrected as a cancer therapy strategy within the last dozen years (8). To target tumor cells and spare normal cells, most of the oncolytic HSV vectors have deletions/mutations in one or more of the genes affecting neurovirulence (*UL56* and *γ34.5*), replication in nondividing cells (*UL39*), or inhibition of protein kinase R pathway activation (γ34.5; ref. 9). Over 20 different oncolytic HSV vectors have been evaluated in a large variety of different tumor types and models (9). Among these 1716, G207, and NV1020 have been translated to the clinic for the treatment of melanoma, malignant glioma, and metastatic colorectal cancer (9). In these studies, we compared the efficacy of G207, its derivative G47 Δ, NV1023, and its IL-12 expressing derivative NV1042 in the C3(1)/T-Ag transgenic breast cancer model (Table 1).

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Table 1. Summary of oncolytic HSV vector efficacy on M6c tumors from C3 (1)/T-Ag mice

Various oncolytic HSV vectors have been tested in both human xenograft and mouse syngeneic models of breast cancer, usually with metastatic disease (38, 40–42). For example, intratumoral injection of murine 4T1 primary tumors with HSV-1 1716 or Synco-2D resulted in a significant reduction in lung metastases (41, 42) . The efficacy of G207 and NV1020, the parental viruses of G47Δ and NV1023,

have been previously compared in a number of different tumor models, including human pancreatic, gastric, and prostate cancer, and mouse bladder and colorectal cancer (28, 43–46). In most cases, both viruses were similarly effective, and the differences seemed to be tumor cell and not tumor type specific. For example, with human head and neck squamous cell carcinoma cell lines, both G207 and NV1020 caused complete regression of nearly all s.c. SSC15 tumors, whereas G207 was ineffective at inhibiting s.c. SCC1483 growth (47), whereas NV1020 was very efficacious (26). Here we found that both G207 and NV1023, derived from NV1020, were ineffective at inhibiting s.c. M6c tumor growth, and G207 was not examined further in the C3(1)/T-Ag transgenic model.

The tumor environment, including surrounding normal cells and extracellular matrix, plays an important role in tumor growth (48) and therapeutic efficacy (49). Differences between the brain parenchyma and the s.c. space may have contributed to the efficacy of NV1023 in treating intracerebral tumors and not s.c. tumors. For many biological therapeutic strategies, the complex stromal-tumor interactions that develop during tumor progression and the generation of immune tolerance against tumor antigens are important features that are not fully operative in implanted tumor models. Genetically engineered mice that spontaneously develop tumors are some of the most representative models we currently have for human cancer, both in furthering our understanding of disease progression and for the preclinical evaluation of new therapies (50). Unfortunately, these tumor models are much more difficult to work with and successfully treat than typical implant models.

In the C3(1)/T-Ag transgenic mouse, the C3(1) regulatory region drives expression of SV40 large and small T-Ag in mammary epithelial ductal cells and the terminal ductal lobular unit (33, 35). Whereas SV40 T-Ag is not expressed in human breast cancer, it inactivates the p53 and Rb pathways that are commonly mutated in breast cancer (51). Heterozygous female C3(1)/T-Ag develop mammary adenocarcinomas, which histologically resembles human breast cancer usually classified as infiltrating ductal carcinoma (34). Although the C3(1) regulatory region contains hormone response elements (52), T-Ag expression is not estrogen responsive. The mouse tumors have up-regulated expression of TGF-α, Her2/*neu*, and c-*myc* and loss of ER-α expression (35). In patients, low ER-α expression is associated with less differentiated, more aggressive, and more difficult to treat tumors. In our studies, we found an incomplete penetrance of tumor formation, with <80% of mice developing tumors, as opposed to the 100% reported in the literature (34), which may reflect epigenetic factors. Genetic polymorphisms may also play a role, as a recent report described the loss of tumor development after breeding on the C57BL background (53).

The availability of tumor cell lines from the C3(1)/T-Ag mice further enhances their utility, providing the means to perform studies *in vitro* and to rapidly screen agents in syngeneic implant models. Tumor cell lines M6 (derived from a spontaneous mammary carcinoma) and M6c (derived from a lung metastasis of an implanted s.c. M6 tumor) both retain expression of the oncogenic transgene (T-Ag; ref. 36), like the spontaneously arising tumors *in vivo* (33). Therefore, they only form tumors in immune-deficient or heterozygous transgenic mice, with tolerance to T-Ag. When M6c cells were implanted into nontransgenic parental FVB/N mice by mistake, no tumors were formed. We found that the M6c cells were more susceptible than the M6 cells to oncolytic HSV replication *in vitro* and that the M6 cells had

highly variable rates of s.c. tumor growth *in vivo*. In addition, the M6c cells formed multifocal tumors when implanted into the brain, providing a model for metastatic breast cancer to the brain. In all three C3(1)/T-Ag cell lines, G47Δ was more cytotoxic than G207 at low MOI, whereas at high MOI G207 was able to kill both the M6c and M6 cells. This enhanced cytotoxicity *in vitro* of G47Δ at low MOI is seen in most tumor cell lines tested (19).[1]

The C3(1)/T-Ag transgenic mouse has proven to be a useful model of breast cancer for the evaluation of experimental therapeutics and chemopreventive agents. A range of chemopreventive agents (retinoids, difluoromethylornithine, dehydroepiandrosterone, and nonsteroidal anti-inflammatory drugs) have been shown to inhibit tumor development, most likely at the progression to invasive carcinoma stage (54). The goal of our studies was to treat established carcinomas that were palpable by direct intratumoral injection. To our knowledge, this is the first description of virotherapy in a transgenic spontaneously arising tumor model. The studies were confounded by the highly variable rates of measurable tumor growth and development of multiple tumors. We tried three different experimental paradigms to accommodate the variable number and time of initiation of tumors. In the first, only the first palpable mammary tumor to arise was treated and all tumors followed for growth. Alternatively, all tumors were treated when they became palpable. We did not detect any effect of G47Δ treatment on the growth of nontreated tumors, or the time of appearance of subsequent tumors, or on the total number of tumors. We have not tried to optimize therapeutic efficacy by altering the treatment paradigm, but based on previous studies in other models, this should be possible.

IL-12 is a proinflammatory cytokine at the intersection of innate and adaptive immunity that has broad antitumor activities, including inducing IFN-γ production, which up-regulates MHC class I and chemokine (IP-10 and MIG) expression, nitric oxide production, and inhibition of angiogenesis; differentiating $CD4^+$ Th1 cells and inducing opsonizing antibodies; and activating CTL and NKT cells (55). We and others have shown that IL-12 expression is very effective at augmenting the antitumor efficacy of oncolytic HSV vectors. IL-12 has been given as a recombinant protein in combination with G207 (56), expressed from a defective HSV vector in combination with G207 (31), or as a transgene encoded by the oncolytic vector (30, 32), as described here. The enhanced efficacy of NV1042 over NV1023 in squamous cell carcinoma was dependent upon $CD4^+/CD8^+$ lymphocytes (30). We found that the effect of local IL-12 expression varied depending upon tumor location. NV1042 ($IL\text{-}12^+$) was more efficacious than NV1023 in the periphery (s.c. and autochthonous), but less in the brain, possibly due to its immune privileged status. In contrast, M002, another oncolytic HSV vector expressing IL-12, significantly extended the survival of mice bearing intracerebral Neuro2a tumors in A/J mice when compared with its parent virus (32).

Administration of rIL-12 with weekly doses of rIL-2 (pulse IL-2) to C3(1)/T-Ag transgenic mice with palpable or multifocal tumors resulted in inhibition of tumor growth, tumor regression in mice with smaller tumor burden, and decreased tumor number (57). However, tumors arose after cessation of treatment, indicating that an effective memory immune response was not generated, and treatment of juvenile mice delayed the appearance of tumors, but did not block their development (57). IL-12 in this system was suggested to be inhibiting angiogenesis, rather than enhancing an immune response. In

contrast, rIL-12 did not inhibit angiogenesis or autochthonous tumor growth in a MMTV-induced mammary carcinoma model, whereas it did with implanted Mm5Mt cells (established from a mouse mammary tumor virus–induced mammary carcinoma; ref. 58), further illustrating the difference between *in situ* arising and implanted tumors. We have not examined the effect of oncolytic HSV or IL-12 on angiogenesis, but NV1042 was recently reported to have antiangiogenic activity in a squamous cell carcinoma model (59).

Direct antiangiogenic factors have also shown significant efficacy in inhibiting mammary tumor growth in C3(1)/T-Ag transgenic mice. Both recombinant mouse endostatin and the human P125A mutant, and VEGF-DT385 toxin inhibited tumor growth and number, and extended survival when given before tumor appearance, although all mice succumbed to disease (60–62). In a gene therapy strategy, i.v. delivery of adenovirus vectors expressing mouse endostatin inhibited cumulative tumor volume, but to a lesser extent than recombinant endostatin (63). Other transgenic mouse breast cancer models have also been used to test various therapeutic agents, again, usually before tumor appearance. The MMTV-neu transgenic mouse has been used to test a variety of antiestrogen and antiangiogenic factors (including plasmids encoding angiostatin, endostatin, TIMP-2, sFLT-1, and IFN-α) and combinations of them (64). The combination of tamoxifen with rIL-12 was very effective at preventing carcinoma development in the Her2/*neu* transgenic mouse (65), likely due again to the antiangiogenic properties of IL-12.

Oncolytic viruses in general and HSV vectors in particular have been applied to the treatment of a large variety of cancers; however, there has been less experimentation with breast cancer. Primary breast tumors can be successfully treated by surgery if they are localized. Unfortunately, many tumors are not caught at an early stage and metastatic and/or hormone insensitive disease ensues, for which treatment is much less effective. C3(1)/T-Ag transgenic mice provide an excellent model for the development and testing of novel therapeutics. While the spontaneous adenocarcinomas are extremely difficult to treat pharmacologically and are non-immunogenic in syngeneic hosts, similar to the clinical situation, we have shown that the third generation oncolytic HSV vector G47Δ has efficacy both in established brain metastases and spontaneous primary tumors, and the IL-12 expressing vector NV1042 was the most effective vector in the periphery.

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Footnotes

Note: R. Liu is currently at the first affiliated hospital of Sun Yat-sen University, Guangzhou, P.R. China. S.D. Rabkin is a consultant to MediGene AG, which has a license from Georgetown University to commercialize G207.

[1] Unpublished data.

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EXPERIMENTAL STUDIES

Reduction and Elimination of Encephalitis in an Experimental Glioma Therapy Model with Attenuated Herpes Simplex Mutants that Retain Susceptibility to Acyclovir

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MALIGNANT GLIOMAS ARE the most common malignant brain tumors and are almost universally fatal. A genetically engineered herpes simplex virus-1 mutant with decreased neurovirulence, *dl*sptk, has been shown to kill human glioma cells in culture and in animal models. However, intracranial inoculation of *dl*sptk is limited by fatal encephalitis at higher doses. Therefore, additional engineered and recombinant herpes simplex mutants with demonstrated reduced neurovirulence (AraA^{r}9, AraA^{r}13, RE6, R3616) were examined as antiglioma agents. One long-term human glioma cell line and two early-passage human gliomas in culture were destroyed by all four viruses tested. In a subcutaneous glioma model, AraA^{r}13, RE6, and R3616 retained substantial antineoplastic effects in nude mice when compared with controls (one-sided Wilcoxon rank test, $P < 0.05$ for one or more doses each). When tested in a nude mouse intracranial glioma model, both RE6 and R3616 significantly prolonged average survival without producing premature encephalitic deaths at two doses (log-rank statistic, $P < 0.007$). Histopathological studies of the brains of surviving animals revealed minimal focal encephalitis in two of three RE6-treated animals and no evidence of encephalitis in either one of three RE6-treated or in three of three R3616-treated animals. No evidence of residual tumor was seen in four of the six surviving animals. Additionally, both RE6 and R3616 were found to be susceptible to the common antiherpetic agent acyclovir, adding to their safety as potential antiglioma agents. Recombinant and engineered viruses that minimize host toxicity and maximize tumoricidal activity merit further study as antineoplastic agents. (Neurosurgery 32:597–603, 1993)

Key words: Brain neoplasm, Encephalitis, Genetic engineering, Glioma, Herpes simplex

Tumors of glial cell origin are the most common primary tumor of the human brain (18, 27). Glioblastomas are the most malignant glioma, comprising one-third of all primary brain tumors, or 5000 new cases annually in the United States (20). Despite surgery, radiotherapy, and chemotherapy, glioblastomas are nearly always fatal, with a median survival rate of less than a year and 5-year survival rates of 5.5% or less (9, 16, 18, 20, 23, 25, 27, 28, 35). Neurological dysfunction and death arise from invasive local growth; systemic metastases are rare (14). No therapeutic modality has substantially changed the natural history and relentless progression of glioblastoma (9, 16, 20, 23, 25, 28, 35).

Recently, we examined a novel experimental therapy for malignant gliomas using a genetically engineered herpes simplex virus (HSV), *dl*sptk (21). *dl*sptk, a thymidine kinase (TK)–negative mutant of HSV-1, is attenuated for neurovirulence because of a 360-base-pair deletion in the TK gene, the product of which is necessary for normal viral replication (8). This deletion compromises replication in nondividing cell populations in the mammalian nervous system (11, 12, 32) while allowing normal replication of virus in rapidly dividing cell populations (15), including neoplasms. Our previous study demonstrated that *dl*sptk treatment was effective in killing human malignant glioma monolayers in culture as well as in slowing subcutaneous glioma tumor growth in athymic (nude) mice (no subcutaneous tumor growth inhibition occurs with

heat-inactivated virus—unpublished data) (21). Additionally, *dl*sptk treatment prolonged overall survival in nude mice with intracranial human glioma xenografts.

However, at both doses tested in the intracranial model (10^3 and 10^5 plaque forming units [pfu]), some animals died, presumably of viral encephalitis, before any tumor-related deaths in the control group (hereafter referred to as premature encephalitic deaths). Subsequent study demonstrated that, even in immunocompetent CD-1 mice, the 50% lethal dose of intracranially administered *dl*sptk is approximately 10^6 pfu (unpublished data). This residual neurovirulence limits the use of larger inocula to improve the tumor cure rate.

Although *dl*sptk retains sensitivity to certain antiviral drugs (foscarnet and vidarabine) (21), potentially allowing antiviral treatment of encephalitis, we hypothesized that other HSV mutants exhibiting decreased neurovirulence might retain cytopathic effects in glioma cells. Such mutants could allow treatment with higher viral doses with reduced toxicity, rendering treatment more effective. Additionally, *dl*sptk is relatively resistant to the favored antiherpetic agents acyclovir and ganciclovir (6) because of its TK deficiency. Such resistance would be a disadvantage should any virus ever achieve clinical use.

Four different HSV mutants were chosen which were known to replicate well in cultured cells but which demonstrate decreased neurovirulence. Two, AraA^{r}9 (8, 10, 12) and AraA^{r}13 (12), contain point mutations in the gene encoding viral DNA polymerase. These mutants are hypothesized to be replication compromised in the brain, because of a lower affinity for deoxynucleoside triphosphates, which are thought to be at low concentration in the brain. Another mutant, RE6 (33, 34), kindly provided by R. Thompson, is an intertypic recombinant (HSV-1 and HSV-2) with at least two lesions conferring attenuation, the more important of which maps to the inverted repeat in the long segment of the HSV genome. The mechanism of decreased neurovirulence seen with RE6 is uncertain. The final mutant, R3616 (4), kindly provided by J. Chou and B. Roizman, contains mutations in both copies of the γ-34.5 gene, which lie in the same repeat sequence. The decreased neurovirulence of R3616 is putatively related to the cessation of neuronal protein synthesis, which is preempted in wild-type HSV infection (5).

To investigate these four mutants as potential antiglioma agents, we conducted assays of glioma cytotoxicity in cell culture, using one long-term human glioma cell line, U87, and two early-passage human glioblastomas. To evaluate tumor inhibition in vivo, subcutaneous U87 xenografts in nude mice were treated separately with inoculations of each viral mutant or vehicle, and tumor growth rates were analyzed. To investigate the potential effects of HSV mutant treatment on survival, nude mice with intracranial U87 xenografts were treated with virus or vehicle inoculations, and overall survival was compared. To evaluate the degree of tumor eradication, as well as the potentially retained neurovirulence of the viruses when used at doses necessary to achieve prolonged survival, the brains of long-term survivors with intracranial xenografts were sectioned, stained, and microscopically examined. Finally, to establish the relative safety of these viruses as potential antiglioma agents, their susceptibility to the common antiherpetic agent acyclovir was investigated.

MATERIALS AND METHODS

Cell line and tumor explants

The malignant human glioma line U87 (American Tissue Cell Collection, Camden, NJ) was used for the cell culture assay; additionally, two primary human malignant gliomas were obtained as surgical tumor specimens, after consent was obtained under the guidelines of the Massachusetts General Hospital Subcommittee on Human Studies, and were started in culture. All cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics (DMEM+). Techniques and maintenance were as described previously (21).

Viruses

HSV mutants were obtained from R. Thompson, B. Roizman, J. Chou, and one of the present authors (D. M. Coen). Procedures involving viruses were in accordance with Harvard Office of Biological Safety guidelines. Viral inoculation and subsequent care of animals harboring a virus were done in approved, designated viral vector rooms. For cell culture assays, viruses (grown on Vero cells as previously described [21]; to maximize the titer of R3616, the initial viral suspension was centrifuged at 34,500 *g* for 2 h at 4°C, and the pellet was subsequently suspended in media and again titered) were applied at two multiplicities of infection (MOIs), 10^{-1} and 10^{-3}.

Cell culture assay of cytotoxicity

MOI values were calculated from cell number as previously described (21). The appropriate number of viral pfu was applied and distributed evenly (6). All viral-infected plates were compared with control plates (DMEM+ only, no virus). Cells were then maintained as described elsewhere (21) and observed microscopically; cells that had become rounded, losing normal morphology, and those lifting from the plate were considered dead. Monolayers were considered completely destroyed when 99% or more of the cells exhibited such cytopathic effects.

Animals

Animal studies were done in accordance with guidelines by the Massachusetts General Hospital Subcommittee on Animal Care. Female nude mice (NCr/Sed nu/nu) were obtained from the Edwin L. Steele Laboratory for Radiation Biology at the Massachusetts General Hospital. Nude mice were anesthetized with 0.25 ml of a solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbitol injection (1 mg/ml), and 6% ethyl alcohol. Animals dying within 48 hours of any procedure were considered perioperative deaths and were excluded from analysis. Deaths in the subcutaneous tumor experiments were excluded from analysis (no significant difference in deaths occurred between virus-treated groups and their corresponding controls).

Subcutaneous glioma xenograft transplantation and therapy

Subcutaneous xenograft implantation was performed as previously described (21). To test the effects of these HSV mutants

on human gliomas in vivo, 1-mm³ minced glioma pieces (obtained from nude mice previously injected subcutaneously with cultured U87 cells) were implanted subcutaneously into nude mice. Between weeks 4 and 5, growing tumors (≥8 mm in diameter) had developed, and mice were divided into two groups of 7 to 10 animals per group. Controls received intraneoplastic injections of 50 or 60 μl of DMEM+; treated animals received similar intraneoplastic injections of virus suspended in DMEM+. Doses administered for each virus are listed in Table 1. Care was taken to distribute virus throughout the tumor. For two-dose experiments, subsequent injections of DMEM+ or virus were made on Day 14. Similar experiments were conducted for each of the four virus mutants at various doses.

Subcutaneous glioma tumor measurements

Tumors were measured weekly (RE6, R3616) or twice weekly (AraA^{r}9, AraA^{r}13) with vernier calipers as previously described (21). Growth of subcutaneous xenografts was recorded as the tumor growth ratio by the formula $([l \times w \times h]/2)/([l \times w \times h]_{day0}/2)$ as previously described (21). Growth ratio comparisons were made at 28 days after the initial treatment except for those for AraA^{r}9, which were made at Day 24 (because of tumor burden deaths). Potential differences in growth ratios were assessed by use of the one-sided Wilcoxon rank test.

Intracranial glioma xenograft transplantation and therapy

To evaluate the safety and efficacy of each virus in treating intracerebral gliomas, nude mice were stereotactically inoculated in the right frontal lobe with 2×10^5 or 4×10^5 U87 glioma cells. For treatment with AraA^{r}9 and AraA^{r}13, animals were randomly divided into control and treatment groups after tumor implantation. Ten days later, control animals received intracranial inoculations of 2 μl of DMEM+; treated animals received intracranial inoculations of 10^5 pfu of selected virus suspended in 2 μl of DMEM+. Group sizes ranged from 8 to 13 animals for all intracranial experiments.

For treatment with RE6 and R3616, a similar protocol was followed except that animals were randomly divided into three groups. Ten days after tumor implantation, the control group received intracranial inoculations of 6 μl of DMEM+ as described above, the second group received intracranial inoculations of 10^5 pfu (low dose) of the test virus, and the third group received intracranial inoculations of 10^7 pfu (high dose) of the test virus, each suspended in 6 μl of DMEM+. Higher doses were attempted with these two viruses because earlier reports (4, 33, 34) demonstrated no fatalities in mice without tumors receiving intracranial inoculations at these doses.

Survival analysis of intracranial investigations

Survival analysis was done as previously described (21). Potential differences in survival were analyzed by use of the log-rank statistic.

Histopathological analysis of intracranial xenograft survivors

After 120 days, all six surviving animals with intracranial xenografts were killed (high-dose, RE6-treated and low-dose, R3616-treated animals, two each; low-dose, RE6-treated and high-dose R3616-treated animals, one each). All appeared neurologically normal at this time. Their entire brains were then fixed, serially sectioned at 10-μm intervals (step sections before and after the lesion), stained with hematoxylin and eosin, and microscopically examined (19).

Determination of herpes simplex virus mutant susceptibility to common antiherpetic agents

Previous studies have demonstrated AraA^{r}9 and AraA^{r}13 to be relatively resistant to acyclovir, although AraA^{r}13 is susceptible to ganciclovir (7). To determine the potential susceptibility of RE6 and R3616 to these agents, plaque reduction assays were conducted as described previously (6). Susceptibility comparison was made to the wild-type HSV stain KOS.

TABLE 1. Viral Inhibition of Subcutaneous U87 Tumor Growth[a]

Mutant	Dose (pfu)	Doses Given	Growth Ratio Comparison	Statistical Significance
AraA^{r}9[b]	5×10^6	Days 0, 14	0.79	NS
AraA^{r}13	10^7	Days 0, 14	0.94	NS
	2.5×10^7	Days 0, 14	0.40	$P < 0.005$
RE6	10^7	Days 0, 14	0.33	$P < 0.05$
	10^8	Day 0 only	0.42	$P < 0.05$
R3616	10^7	Days 0, 14	0.75	NS
	10^8	Day 0 only	0.47	NS
	2×10^9	Days 0, 14	0.47	$P < 0.05$
*dl*sptk	10^7	Days 0, 14	0.21	$P < 0.05$

[a] Mice were injected intraneoplastically with virus suspended in 0.05 ml of Dulbecco's modified Eagle's medium with fetal bovine serum and antibiotics. Growth ratio is a measure of the rate of tumor growth; see Text for method of calculation. Growth ratio comparisons are the ratio of the treated group growth ratio mean to the control group growth ratio mean; a value of less than one demonstrates tumor growth inhibition. Experiments could not be carried out past 28 days because of tumor burden deaths in control animals. Statistical significance was calculated by one-sided Wilcoxon rank test. Values greater than 0.05 are listed as not significant (NS); the exact test score was computed for values near the level of significance. For comparison, parallel *dl*sptk studies are shown. pfu, plaque-forming units.

[b] Study discontinued at 24 days because of tumor burden deaths.

RESULTS

Cell culture cytotoxic assays

All viruses destroyed the glioma monolayers via spreading infection at both MOIs tested, with time to complete monolayer destruction ranging from 3 days for AraA^{r}9 at an MOI of 10^{-1} to 16 days for R3616 at an MOI of 10^{-3}.

In vivo xenografts: Subcutaneous

At the highest inoculum available (5×10^6 pfu), AraA^{r}9 never demonstrated effective inhibition of tumor growth; a short-term study of *dl*sptk at this dose showed statistically significant reduction of tumor growth within 14 days (data not shown), and after 1 month, when 10^7 pfu were inoculated on days 0 and 14. The remaining three mutants tested all showed significant tumor growth inhibition, although some required higher doses than *dl*sptk (Table 1). To determine if one dose was adequate to inhibit tumor growth, RE6 and R3616 were tested at 10^8 pfu; only growth inhibition by RE6 reached significance.

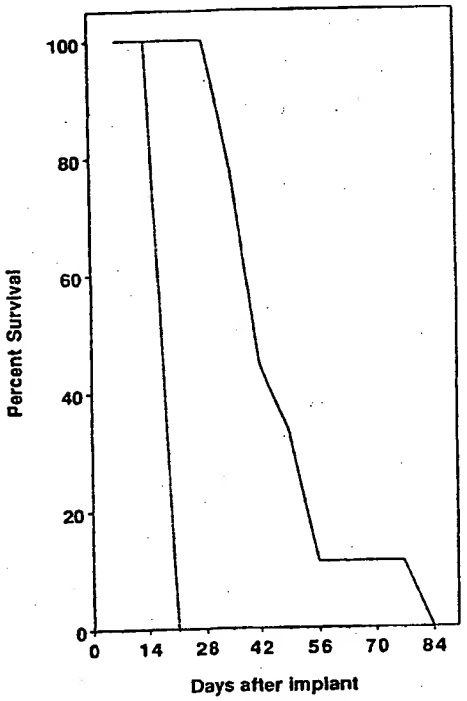

FIGURE 1. Stereotactic intracranial injections of U87 cells were made into the right frontal lobe of nude mice. Ten days later, control mice received stereotactic intratumoral injections of medium; treated animals received medium containing mutant virus. Shown are percentages of animals surviving for mice treated with the virus AraA^{r}9 compared with controls. *Solid lines* represent survival in the control groups, and *dotted lines* represent survival in animals treated with 10^5 pfu of virus.

In vivo xenografts: Intracranial

Mice treated with intracranial inoculations of 10^5 pfu of AraA^{r}9 all died within 11 days of virus treatment (21 days after implantation), whereas untreated controls lived a median of 45 days after implantation (Fig. 1). Mice treated with AraA^{r}13 also demonstrated a decrease in median days of survival when compared with controls, without a significant increase in long-term survivors (Fig. 2).

Statistically significant increases in survival were seen in all four groups of RE6 and R3616 virus-treated animals compared with controls (median survival was 42 d after tumor implantation for control animals for all experiments): RE6: 10^5 pfu, median survival, 58 d (log-rank statistic, $P < 0.004$; Fig. 3); RE6: 10^7 pfu, median survival, 84 d (log-rank statistic, $P < 0.007$; Figure 3); R3616: 10^5 pfu, median survival, 71 d (log-rank statistic, $P < 0.0003$; Fig. 4); R3616: 10^7 pfu, median survival, 63 d (log-rank statistic, $P < 0.0003$; Fig. 4). There were no statistically significant differences in survival among treated groups. In none of the four treated groups did any deaths occur before those in the control group; percent survival in each of the treated groups was at all times greater than or equal to percent survival in the control group.

Histopathological analysis

Small tumor masses (approximately 5 mm or less in diameter) were present in the brain treated with low-dose RE6 and in one of the brains treated with low-dose R3616, but no neo-

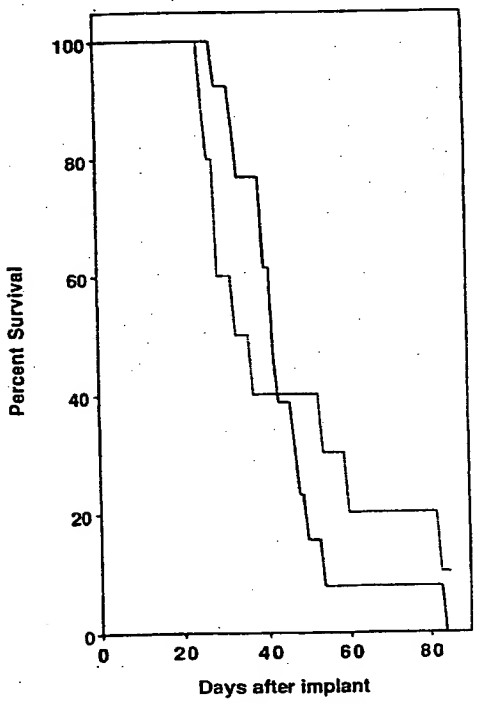

FIGURE 2. Percentages of animals surviving for mice treated with AraA^{r}13 compared with controls. For details, see legend to Figure 1.

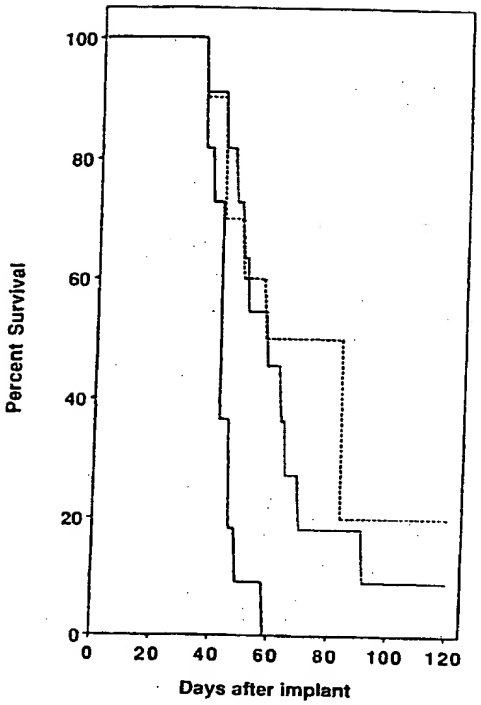

FIGURE 3. Percentages of animals surviving for mice treated with RE6 compared with controls. For details, see legend to Figure 1. Dashed lines represent additional treatment groups each receiving viral doses of 10^7 pfu.

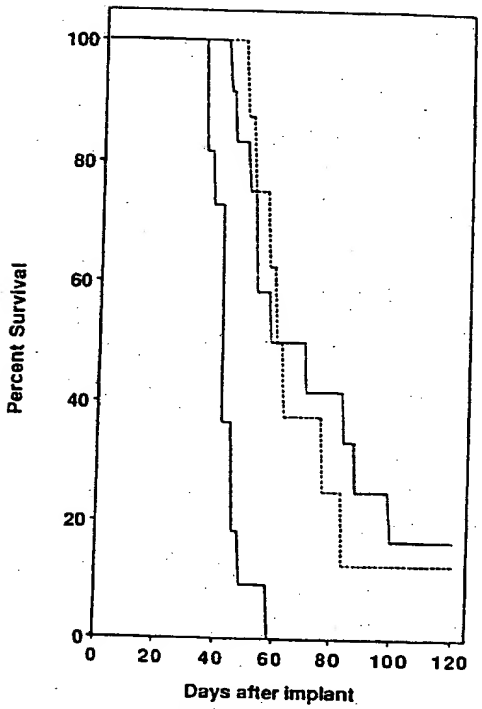

FIGURE 4. Percentages of animals surviving for mice treated with R3616 compared with controls. For details, see legend to Figure 1. Dashed lines represent additional treatment groups each receiving viral doses of 10^7 pfu.

plastic cells were seen in the remaining low-dose, R3616-treated brain. No tumor cells were seen in the brains of mice receiving the high dose of either virus.

A potential major drawback seen in *dl*sptk therapy was the presence of widespread meningoencephalitis in the long-term surviving animals (21). Sections from the brains treated with RE6 and R3616 showed no evidence of widespread disease; two of the brains treated with RE6 showed only scattered vasculitis, occasional microglial nodules, and/or mild meningitis. No evidence of encephalitis or necrosis was present in the R3616-treated brains at either dose or in the other (high-dose) RE6-treated brain. No herpetic inclusion bodies were present in any of the brains, nor were any present in the residual tumor tissue when present.

Antiherpetic agent susceptibility

AraA^{r}13, although somewhat resistant to acyclovir, is susceptible to ganciclovir (6). Both RE6 and R3616 were as susceptible to acyclovir as the herpes wild-type virus KOS when tested via plaque reduction assay (data not shown).

DISCUSSION

The use of wild-type or attenuated viruses for the treatment of both animal and human tumors has been attempted in the past (1–3, 17, 22, 24, 26, 29–31). Direct cell killing by the virus or the production of new antigens on the tumor cell surface with subsequent immunological rejection was the proposed mechanism of action. However, in all of those earlier studies, wild-type virus, passage-attenuated virus, infected cell preparations, or isolated mutants were used. Treatment failure generally resulted either from systemic viral illness or inadequate antineoplastic effects. Before our initial work with *dl*sptk, genetically engineered viruses had not been tested.

As we demonstrated earlier with *dl*sptk (21), and have confirmed with this investigation, recombinant and genetically engineered viruses can be constructed to destroy glioma cells both in culture and in vivo. Whereas our previous study demonstrated inhibition of tumor growth and increased survival in intracranially implanted tumors treated with a TK-negative HSV, this study confirms that inhibition of tumor growth can be achieved with mutants retaining TK proficiency. However, careful choice of mutant is essential for effective in vivo tumor inhibition and survival prolongation; in this study, subcutaneous tumor growth was significantly inhibited by only one of the deoxyribonucleic acid polymerase mutants (AraA^{r}13), whereas neither deoxyribonucleic acid polymerase mutant prolonged survival.

Second, this study demonstrates that early encephalitic deaths, chronic encephalitis, and brain necrosis can be eliminated with selected mutants. An additional safety advantage of the TK-proficient mutants RE6 and R3616 over *dl*sptk is re-

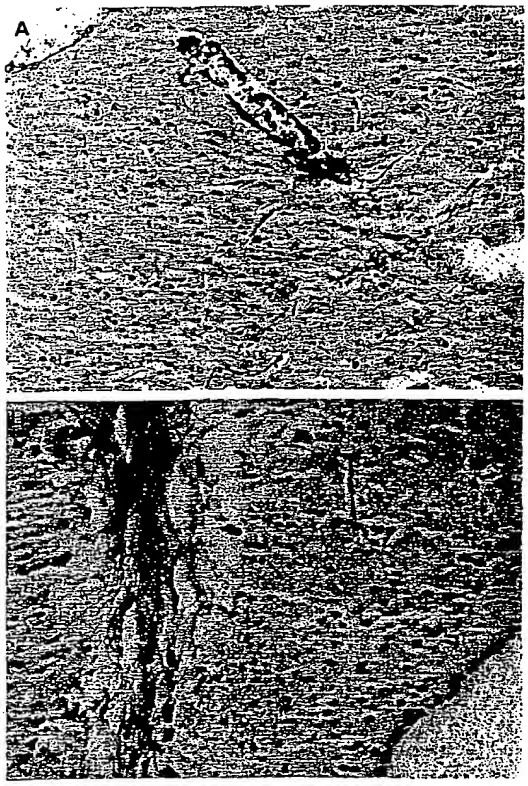

FIGURE 5. Shown are representative 10-μm sections from brains of long-term survivors (120 d) of intracranial U87 injections, treated with 10^5 *pfu of RE6 (A)* and 10^7 pfu of R3616 (*B*). Similar doses of wild-type HSV cause 100% mortality from encephalitis within 4 weeks, whereas long-term surviving mice treated with 10^5 pfu of *dl*sptk showed widespread meningoencephalitis in an earlier study. In the RE6-treated brain (magnification, ×100) are residual U87 tumor cells (not shown), as well as scattered, mild meningitis (meningeal inflammation, *upper left*), intracortical microglial nodules (*right center*), and perivascular inflammation (*top center*). The R3616 section (magnification, ×175) shows normal striatum surrounding the needle track (*left*); no evidence of residual tumor, meningoencephalitis, or microglial nodules was seen.

tained susceptibility to acyclovir, the drug of choice for treating HSV (13). Studies are now needed to explore the effects of viruses engineered to contain multiple mutations, which may further reduce neurovirulence and the possibility of wild-type reversion. It will also be important to study immunocompetent animals, including long-term evaluations of active virus and latent virus distribution in tumor and normal brain throughout the time course of infection, as well as potential acute and chronic neuropathological changes before considering the clinical utility of engineered oncolytic viral therapy for the treatment of brain tumors.

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We thank L. A. Pozzi for performing acyclovir sensitivity assays, R. Thompson (University of Cincinnati) for providing RE6 and J. Chou and B. Roizman (University of Chicago) for providing R3616; P. Schaffer for providing Vero (African green monkey kidney) cells; the Massachusetts General Hospital Neurosurgery Service for providing tumor specimens; D. Schoenfeld and K. Kleinman, Massachusetts General Hospital Biostatistics Center, for statistical analysis of the data; and J. P. Vonsattel for neuropathological analysis. Supported by grants from NF Inc. (Massachusetts Bay area) to R. L. Martuza and from the National Institutes of Health (NS24279 to Martuza, and RO1-AI19838, SO7RRO5381, and PO1AI2410 to D. M. Coen).

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COMMENTS

The concept of treating gliomas with a viral agent that can then be eliminated with an antiviral drug has been an intriguing one. The major limitation has been the persistence of a viral infection "encephalitis" after therapy. As dreaded as glioblastoma may be, replacing this terrible tumor with an encephalitic brain lacks any appeal. It is thus extremely exciting to learn of this new work, wherein a particular attenuated herpes simplex mutant has been used to eliminate much of the risk of encephalitis.

A further limitation in this therapy has been that such an "engineered mutant" would lose its ability to respond to the antiviral agent acyclovir. This specific mutant apparently remains sensitive to the action of acyclovir; thus, the approach is safe, and the appeal of this concept is raised significantly.

Obviously, the work described in this article reflects the results in an animal model system; nevertheless, this work suggests that, with properly engineered mutants, a new era of experimental therapies may be opened for detailed study.

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Pittsburgh, Pennsylvania

Markert and colleagues have demonstrated the use of acyclovir-sensitive herpes simplex mutants with decreased encephalitis potential for the treatment of nude mice inoculated with subcutaneous or intracranial malignant glioma. The study demonstrates improved survival and decreased tumor growth for virus-treated animals when compared with controls. The variability in survival between study groups, common in in vivo experiments, points out the importance of postimplantation randomization and specific controls for each experiment.

It will be important to determine the cell-type specificity of normal brain infection by these mutant viruses as well as the time course of infection. It will also be important to know the time course of recoverable active or latent virus from the brain after virus inoculation. Parallel studies should be performed to determine histopathologically the presence of encephalitis at various earlier and later times, preferably using monoclonal or in situ virus probes and antibodies against leukocyte populations.

Successful glioma therapy depends on identifying cytotoxic agents with tumor-specific activity and minimal toxicity to normal brain. The potential of viral or gene therapy for these tumors is promising; however, the need for careful safety studies is essential. The authors' suggestion for the use of viruses with multiple mutations to decrease neurovirulence and prevent wild-type reversion is appropriate.

David R. Hinton
Los Angeles, California

[CANCER RESEARCH 54, 3963–3966, August 1, 1994]

Advances in Brief

Treatment of Malignant Gliomas Using Ganciclovir-hypersensitive, Ribonucleotide Reductase-deficient Herpes Simplex Viral Mutant[1]

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Abstract

We have demonstrated that attenuated mutants of herpes simplex virus (HSV) have therapeutic potential for malignant brain tumors. In this report, we tested a ribonucleotide reductase-deficient (RR^-) HSV mutant as an experimental treatment for malignant brain tumors. The HSV-RR^- mutant hrR3, containing an *Escherichia coli lacZ* gene insertion in the *ICP6* gene that encodes the large subunit of RR, was used in this study. We examined the cytopathic effect of hrR3 (0.1 plaque-forming unit/cell) on the U-87MG human glioblastoma cell line *in vitro*. Only 0.2% of U-87 cells were alive 67 h postinfection. Drug sensitivity assays demonstrated that hrR3 is hypersensitive to the antiherpetic agent ganciclovir. For *in vivo* studies, 10 animals harboring U-87MG tumors were randomly divided and treated intraneoplastically with either 5×10^6 plaque-forming units of hrR3 or medium alone. The viral treatment group showed significant inhibition of tumor growth ($P < 0.01$; one-sided Wilcoxon rank test). Expression of the *lacZ* gene in hrR3, visualized by 5-bromo-4-chrolo-3-indolyl-β-D-galactopyranoside histochemistry, could be detected in treated tumors. The therapeutic potential of this HSV-RR^- mutant for malignant gliomas is discussed.

Introduction

Despite many recent advances in neurosurgical techniques, radiation therapy, and chemotherapy, the prognosis for patients with malignant brain tumors has not improved dramatically. The 5-year survival for glioblastoma multiforme, the most malignant glioma, is still 5.5% or less (1, 2). This led us to examine novel therapeutic approaches utilizing genetically engineered viruses (3–5). Recent experimental studies indicate that engineered viruses derived from HSV-1[3] may have therapeutic potential for the treatment of human malignant gliomas. Previously, we demonstrated that a TK^- mutant *dl*sptk could destroy human malignant glioma cells in an animal brain tumor model (3, 4). The HSV-*TK* gene encodes a key enzyme in the *de novo* synthesis of nucleotide precursors. This deletion compromises DNA replication in nondividing cells, including those in the mammalian nervous system (6–9). We hypothesized that the HSV-TK^- mutants might effectively treat brain tumors while sparing normal brain cells. However, in considering clinical trials of this therapeutic approach, an important issue is the resistance of the HSV-TK^- mutant to commonly used antiherpetic agents such as ACV and GCV. In the central nervous system, the major side effect of using replication-competent viruses as therapeutic agents is the possible production of encephalitis. For example, with *dl*sptk, the 50% lethal dose after i.c. inoculation of CD-1 mice was 10^6 pfu (5). Therefore, although *dl*sptk is sensitive to foscarnet and vidarabine (3), it would be beneficial to use a HSV mutant that is sensitive to GCV or ACV and less neurovirulent.

RR is also a key enzyme in the *de novo* synthesis of DNA precursors, catalyzing the reduction of ribonucleotides to deoxyribonucleotides (10). HSV-1 encodes its own RR, which is composed of two nonidentical subunits (11). The large subunit (M_r 140,000), designated *ICP6*, is tightly associated to the small subunit (M_r 38,000). HSV-RR is required for efficient viral growth in nondividing cells but not in many dividing cells (12–15), indicating that the HSV-RR^- mutant might have therapeutic potential for malignant gliomas while maintaining antiherpetic drug sensitivity. We studied the efficacy of HSV-RR^- mutant hrR3, which contains an *Escherichia coli lacZ* gene insertion in the *ICP6* gene, against malignant human gliomas. The presence of the *lacZ* gene in hrR3 allows identification of virally infected tumor cells using β-galactosidase histochemistry. Furthermore, because this mutant is hypersensitive to GCV, it may be a more clinically useful therapeutic agent for malignant gliomas than HSV-TK^- mutants.

Materials and Methods

Viruses and Cell Lines. HSV-1 wild type strain KOS was kindly provided by Donald M. Coen (Harvard Medical School, Boston, MA). HSV-RR^- mutant hrR3, which possesses the structural gene of *E. coli lacZ* inserted into the RR large subunit (*ICP6*) gene of HSV-1 KOS (13), was kindly provided by Sandra K. Weller (University of Connecticut Health Center, Farmington, CT). Stocks of viruses were generated in African green monkey kidney cell (Vero) cultures as described (3). Virus titration was performed as described elsewhere (3). Human glioblastoma cell lines U-87MG, T98G, U-138MG, and A172 were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's minimal essential medium, supplemented with 10% inactivated fetal calf serum and antibiotics.

Cell Culture Cytotoxicity. Viruses were infected onto subconfluent monolayers of U-87MG cells in 25-cm^2 tissue culture flasks at a MOI of 0.1 pfu/cell (an MOI of 0.1 means that 1 plaque-forming viral particle was added/10 tumor cells), while controls were mock infected. Viable cells were determined by trypan blue exclusion on days 1–4.

GCV Sensitivity Assay. Confluent monolayers of Vero cells in 12-well plates were infected with 100 pfu of KOS or hrR3, where the MOI remains below 0.0005. After the virus inoculum was removed, Dulbecco's minimal essential medium plus 1% inactivated fetal calf serum and 1000-fold diluted human immunoglobulin (Armour Pharmaceutical Company, Kankakee, IL) containing various concentrations of GCV were added to triplicate cultures and cells were incubated at 37°C. Plaques were visualized by Giemsa stain and counted on day 3 postinfection.

Animal Studies. Six-week-old female athymic BALB/c-*nu/nu* mice were purchased from the National Cancer Institute (Rockville, MD) and maintained in our designated animal facilities. All animal procedures were approved by the Georgetown University Animal Care and Use Committee. For surgical procedures, each mouse was anesthetized with an i.p. injection of a 0.25–0.30-ml solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbital (1 mg/ml; Abbott Laboratories, Chicago, IL), and 6% ethyl alcohol.

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[2] To whom requests for reprints should be addressed, at Department of Neurosurgery, Georgetown University Medical Center, 3800 Reservoir Road, N. W., Washington, DC 20007.

[3] The abbreviations used are: HSV-1, herpes simplex virus type 1; TK^-, thymidine kinase deficient; RR^-, ribonucleotide reductase deficient; ACV, 9-[(2-hydroxyethoxy)-methyl]guanine (acyclovir); GCV, 9-[[hydroxy-1-(hydroxymethyl) ethoxy]methyl]guanine (ganciclovir); *ICP6*, infected cell protein 6; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; i.c., intracranial; pfu, plaque-forming unit; i.n., intraneoplastically; HBSS, Hanks' balanced salt solution.

s.c. Glioma Therapy and X-gal Staining. U-87MG tumors were removed aseptically from the flanks of host mice, minced into 1-mm pieces, and transplanted into additional mice for study. Mice harboring s.c. tumors (>6 mm in diameter) were randomly divided (n = 5/group) and treated i.n. with either 5×10^6 pfu of hrR3 virus suspended in 0.05 ml HBSS or with HBSS alone. Treatment was repeated in an identical fashion 10 days later. The tumor diameter was measured by external caliper measurements. Tumor growth ratio was determined as:

$$\frac{l \times w \times h}{(l \times w \times h)_{\text{day 0}}}$$

in which l is length, w is width, and h is height. Statistical differences in growth ratios were assessed by use of one-sided Wilcoxon rank test.

For pathological studies, tumor-bearing mice (>10 mm in diameter) were treated with a single injection of 5×10^6 pfu of hrR3 and sacrificed on days 3, 7, and 14 postinjection. Animals were perfused by 2% paraformaldehyde-5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid-2 mM magnesium chloride in 0.1 M 1,4-piperazinediethanesulfonic acid buffer, pH 7.3. The s.c. tumors were removed, placed in fixative for 1 h, and submerged in cold phosphate-buffered saline. Tumors were then placed overnight in substrate solution (containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40), washed with phosphate-buffered saline, and incubated overnight in cold phosphate-buffered saline containing 30% sucrose and 2 mM magnesium chloride. After being frozen on dry ice, tumors were sectioned on a cryostat. The sections were mounted onto gelatin-coated glass slides and counterstained with hematoxylin and eosin solution.

Results

***In Vitro* Cytopathic Efficacy.** To determine whether a HSV-RR$^-$ mutant could destroy malignant glioma cells, human glioma cell lines U-87MG, T98G, U-138MG, and A172 were infected with hrR3 (MOI, 0.1). All four cell lines were efficiently destroyed by hrR3 within 5 days. With U-87 cells, a cytopathic effect appeared on day 1 postinfection and >99% cytotoxicity was evident on day 3 (Fig. 1). Cells became round, lost normal morphological features, and lifted off the plate. The cytopathic efficacy of hrR3 to U-87MG cells was almost the same as that of *dl*sptk (3). β-Galactosidase expression in hrR3-infected cells was also examined. Infected cells exhibiting a cytopathic effect were stained with X-gal (data not shown).

GCV Hypersensitivity of HSV-RR$^-$ Mutant. We compared the GCV sensitivity of hrR3 with that of the parental wild type HSV strain KOS. hrR3 was approximately 10 times more sensitive to GCV than to KOS, with a median effective dose of 4–5 ng/ml, while the median effective dose for KOS is 40–50 ng/ml (Fig. 2). This is similar to the results of Coen *et al.* with ACV (16).

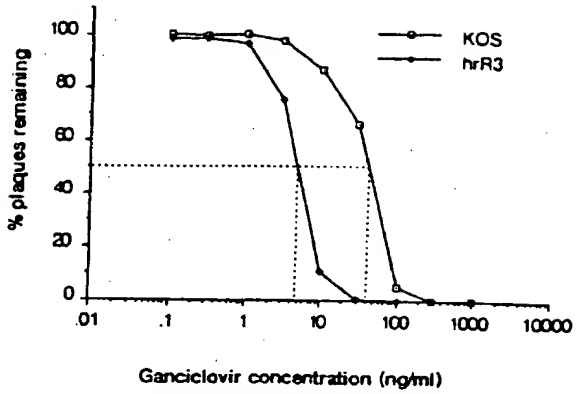

Fig. 2. Ganciclovir sensitivity of hrR3 and KOS. hrR3 and KOS were infected to Vero cells and incubated in media containing various concentrations GCV. The plates were fixed and stained with giemsa and viral plaques were counted. *Points*, means of triplicate wells. The plaque number in the absence of GCV represents 100% plaques. . . . . , median effective dose.

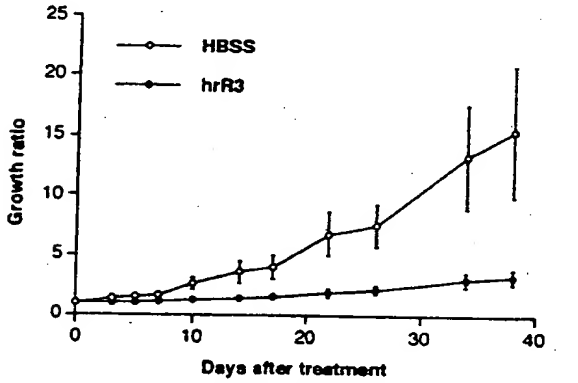

Fig. 3. s.c. U-87MG tumor growth in BALB/c-*nu/nu* mice. Mice harboring s.c. U-87MG tumors (>6 mm in diameter, 3 weeks postimplantation)) were treated with either 5×10^6 hrR3 on days 0 and 10 or control medium (n = 5/group). The mean tumor growth rate was significantly inhibited in hrR3-treated tumors compared to control tumors treated with medium alone.

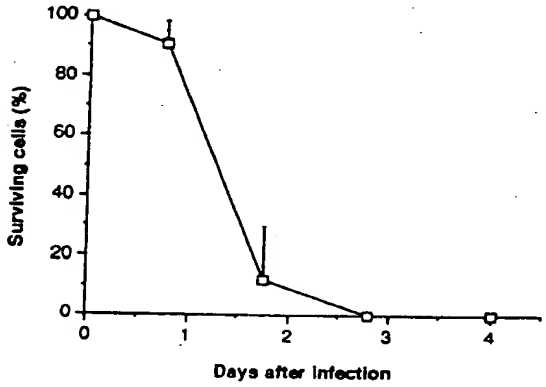

Fig. 1. *In vitro* cytopathic efficacy of hrR3 on U-87MG cells. Duplicate flasks of U-87MG cells were infected with hrR3 at a MOI of 0.1. Viable cells were determined and compared with mock infected cells on days 1–4 postinfection. Cell survivals at 18 and 67 h postinfection are 90.7 and 0.2% of controls. *Points*, means; *bars*, SDM.

***In Vivo* Treatment and X-gal Histochemistry.** We next studied the effect of hrR3 infection on s.c. xenografts. U-87MG tumor fragments were transplanted into BALB/c-*nu/nu* mice. Growing tumors (>6 mm in diameter) were evident by week 3, at which time tumors were treated with i.n. injections of hrR3 or HBSS (control). There was no significant difference in tumor diameter between control and hrR3-treated tumors for the first few days after the first injection of hrR3. From day 10 onward the tumor size between the two groups diverged. When the experiment was terminated on day 38 (Fig. 3) because of tumor size in the control animals, the mean tumor growth rate was significantly inhibited ($P < 0.01$; one-sided Wilcoxon rank test.) in hrR3-treated tumors [growth ratio, 3.19 ± 0.628 (SEM)] when compared to control tumors treated with HBSS alone (growth ratio, 15.4 ± 5.47).

To assess the spread of virus in U-87MG tumors *in vivo*, tumor-bearing mice were sacrificed on days 3, 7, and 14 following treatment. These tumors were fixed and stained with X-gal in order to examine the extent of β-galactosidase expression. On day 3 posttreatment,

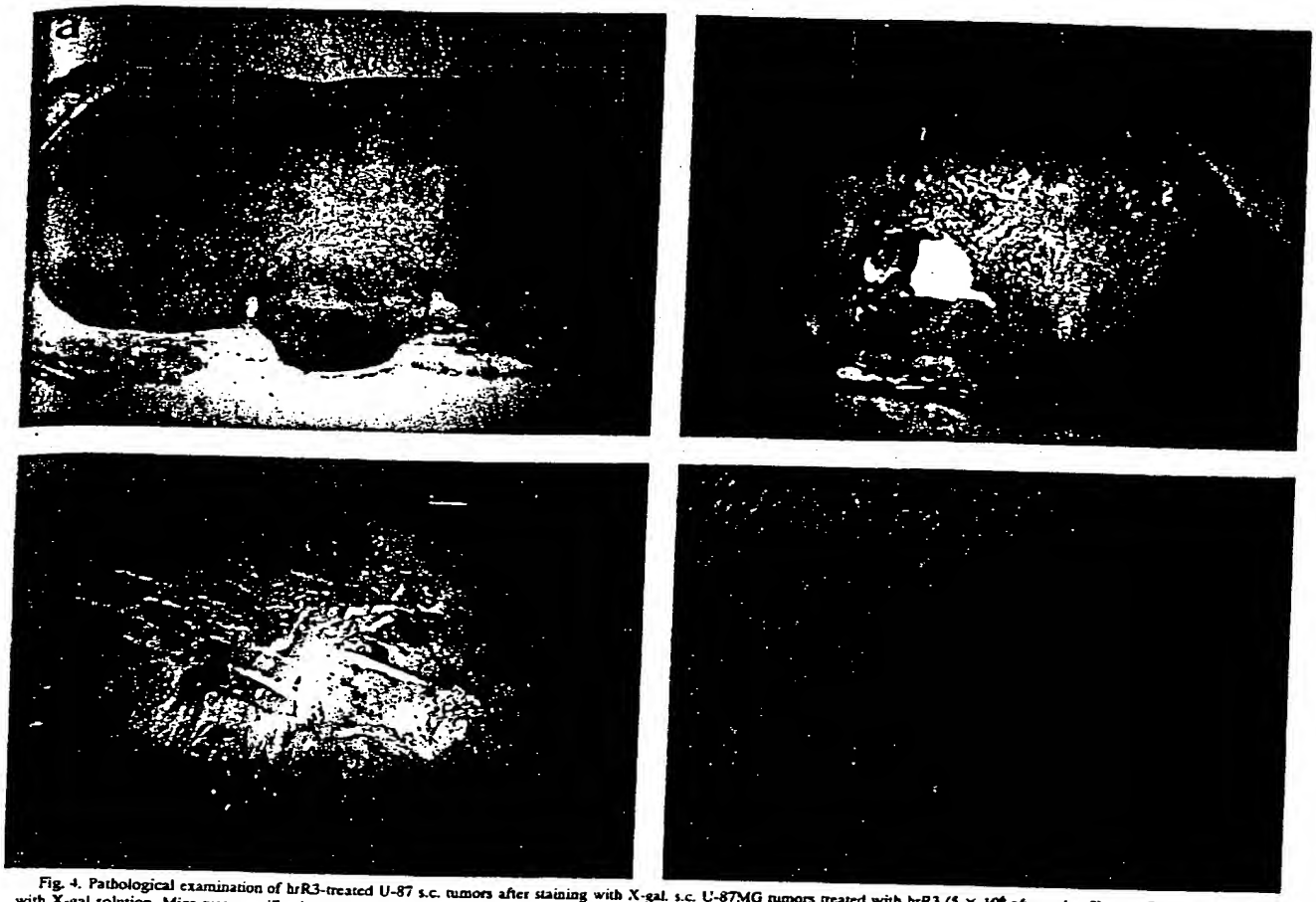

Fig. 4. Pathological examination of hrR3-treated U-87 s.c. tumors after staining with X-gal. s.c. U-87MG tumors treated with hrR3 (5×10^6 pfu on day 0) were fixed and stained with X-gal solution. Mice were sacrificed on days 3 (*a*), 7 (*b*), and 14 (*c* and *d*) postinfection. Micrographs of 20-μm sections are at low (*a*, *b*, and *c*; *bar*, 1 mm) and high (*d*; *bar*, 50 μm) magnification.

positive X-gal staining cells were observed at injection sites and around the periphery of the tumor. This may be due to leakage of inoculum within the tumor capsule and the presence of actively growing tumor cells at the periphery (Fig. 4*a*). Some blue cells were present outside of the tumor capsule but these did not persist at later time points. On days 7 and 14, staining of peripheral cells disappeared and the area of positive X-gal staining within the tumor was expanded, which suggests spread of the virus (Fig. 4, *b* and *c*). At higher magnification, these blue tumor cells appeared necrotic, losing the typical morphological appearance of a U-87MG cell (Fig. 4*d*).

Discussion

Our previous studies demonstrated that HSV-TK$^-$ mutants have therapeutic potential for malignant brain tumors (3, 4). In this study, we examined the effect of HSV-RR$^-$ mutants on malignant glioma cells. Both HSV-TK$^-$ and HSV-RR$^-$ mutants can replicate in dividing cells but not in nondividing cells due to mutations in key enzymes for nucleotide metabolism (3, 6, 13). Our model depends on the ability of HSV mutants to replicate in actively growing glioma cells while sparing normal, postmitotic brain cells and effectively destroying malignant glioma tumors *in vivo* with minimal collateral damage. We are characterizing different viral mutations in order to optimize the efficacy of this approach. In this study, we demonstrate that the HSV-RR$^-$ mutant hrR3 destroyed human U-87MG cells *in vitro* and *in vivo* as well as the HSV-TK$^-$ mutant *dl*sptk.

An important difference between these HSV mutants is that hrR3 is hypersensitive to ACV and GCV while *dl*sptk is resistant. In the case of mutant virus replication outside the tumor, HSV-TK$^-$ mutants are resistant to the most commonly used antiherpetic nucleoside analogues and are therefore currently difficult to treat, whereas HSV-RR$^-$ mutants are hypersensitive to ACV and GCV. Lack of RR leads to reduced deoxynucleotide triphosphate pool synthesis, which could increase inhibition of DNA replication by deoxynucleotide triphosphate-competitive inhibitors such as GCV-TP (16, 17).

Another difference between the behavior of HSV-TK$^-$ and RR$^-$ mutants is temperature sensitivity of viral growth. HSV-RR$^-$ is severely compromised in its ability to produce infections and synthesize viral DNA at 39.5°C *in vitro*, while HSV-TK$^-$ can grow as well as wild type at elevated temperatures (6, 13, 14, 18). The molecular basis for this sensitivity is not clear. In considering clinical trials of HSV-RR$^-$ mutants, this behavior may influence the therapy. Direct inoculation of HSV-RR$^-$ mutants in the tumor may lead to local inflammation and then local fever. The killing activity of the mutants might be decreased *in vivo* due to the elevated temperature. On the other hand, one of the symptoms of encephalitis is high fever. HSV-RR$^-$ mutants in this case might be attenuated for replication in normal

brain. Therefore, this temperature sensitivity of HSV-RR$^-$ could either protect the host or diminish the efficacy of the treatment. We demonstrated that hrR3 significantly inhibited the growth of s.c. U-87MG human malignant glioblastomas as well as *dl*sptk, suggesting that HSV-RR$^-$ mutants could destroy tumor cells effectively *in vivo*.

There are several HSV-RR$^-$ mutants available for therapy (13, 14, 18). In this study, we used hrR3, which contains *E. coli lacZ* gene in its genome. The presence of the *lacZ* gene in the genomic DNA of HSV mutants provides a sensitive means to track not only viral infection within the tumor but also ectopic spread of the virus outside the tumor. We demonstrated positively X-gal-stained U-87MG cells *in vitro* and *in vivo*, suggesting viral replication and spread. Further characterization of viral spread with HSV mutants containing *lacZ* will help to understand the mechanism of HSV tumor therapy.

In order for this therapy to become an effective clinical choice, it is important that different attenuated HSV strains and mutants affecting neurovirulence be examined. A fine balance must be achieved between optimizing the efficacy of tumor cell killing, minimizing spread of the virus in non-tumor tissue, and safety options.

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The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors

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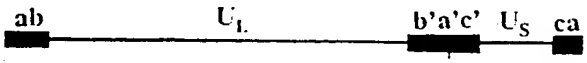
ABSTRACT **Herpes simplex virus vectors are being developed for delivery and expression of human genes to the central nervous system, selective destruction of cancer cells, and as carriers for genes encoding antigens that induce protective immunity against infectious agents. Vectors constructed to meet these objectives must differ from wild-type virus with respect to host range, reactivation from latency, and expression of viral genes. The vectors currently being developed are (*i*) helper free amplicons, (*ii*) replication defective viruses, and (*iii*) genetically engineered replication competent viruses with restricted host range. Whereas the former two types of vectors require stable, continuous cell lines expressing viral genes for their replication, the replication competent viruses will replicate on approved primary human cell strains.**

Herpes simplex viruses (HSV) and particularly HSV-1 are potential vectors for several applications in human health. These include (*i*) delivery and expression of human genes to central nervous system (CNS) cells, (*ii*) selective destruction of cancer cells, and (*iii*) prophylaxis against infections with HSV and other infectious agents. The properties of wild-type virus are fundamentally antithetical to such applications. HSV-1 is highly destructive to infected cells. In addition, HSV-1 is generally spread by contact of the tissues containing virus of one individual with mucous membranes of an uninfected individual. The virus multiplies at the portal of entry, infects sensory nerve endings innervating the site of multiplication, and is transported retrograde to the nucleus of sensory neurons. The sequence of events beyond this point are less well known. In experimental animal systems, the virus multiplies in some neurons but establishes a latent state in others. In a fraction of those infected, the virus periodically reactivates from latent state. In these neurons the newly replicated virus is transported anterograde, usually to a site at or near the portal of entry into the body, where it may cause a localized lesion. In immunosuppressed individuals, the lesions caused both by initial infection and recrudescences tend to be more extensive and persist longer than in immunocompetent individuals (reviewed in refs. 1 and 2). To serve as vectors, the viral genotype must be extensively altered to fit the objective of the vector. For example, to deliver and express human genes in the CNS, the desirable properties of HSV are its ability to establish latent infections and the huge coding capacity of the viral genome. The undesirable property is the capacity of the virus to commit the cell to destruction very early in infection. For selective destruction of cancer cells, the desirable property is the capacity of the virus to destroy cells. The undesirable properties are the wide host range of the wild-type virus and the capacity of the virus to reactivate from latent state. Each application therefore requires a different kind of vector and, in principle, different kinds of genetic engineering. The purpose of this report is to summarize our knowledge of the molecular biology of HSV-1 relevant to experimental design of viral vectors.



Genome Domains	Open Reading Frames	No.
L Component		
ab, b'a'	α0, $\gamma_1$34.5, ORF-P, O	8
U_L	U_L1-56, 8.5, 9.5, 10.5 12.5, 20.5, 26.5, 27.5	
	43.5, 49.5	65
S Component		
a'c', ca	α4	2
U_S	U_S1-12, 1.5, 8.5	14
	Total	89
	Total single copy	84

FIG. 1. Sequence arrangement in HSV DNA and distribution of viral genes in the HSV genome. The filled quadrangles represent terminal sequence ab and ca inverted and repeated internally to yield b'a'c'. See ref. 1 and Table 1 for details of genome structure and gene function. The latency associated transcripts (LATs) map within inverted repeats flanking U_L.

Genome Structure and Gene Content

The genome structure and current gene content of HSV-1 are summarized in Fig. 1. Exclusive of the variable number of repeats of the terminal a sequence, the HSV-1 genome is approximately 152 kbp in size (3, 4). The genome consists of two long stretches of quasi-unique sequences, unique long sequence (U_L) and unique short sequence (U_S), flanked by inverted repeats. U_L is flanked by the sequence ab and its inversion b'a', approximately 9 kbp each, whereas U_S is flanked by the sequence a'c' and its inversion ca, 6.5 kbp each (5, 6). Thus, the HSV genome contains 15 kbp of DNA sequences (b'a'c'), which represent inverted repeats of terminal regions inserted between U_L and U_S domains. The a sequence varies in size and may be present in multiple copies adjacent to the ba sequence, but only in a single copy at the

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Abbreviations: HSV-1, -2, herpes simplex virus 1 and 2; ICP, infected cell protein; gB, gC, gD, etc., glycoproteins B, C, D, etc.; U_L, unique long sequence; U_S, unique short sequence; LAT, latency associated transcripts; ORF, open reading frame; CNS, central nervous system.

Table 1. The function of herpes simplex virus genes

Gene	Product	Dispensable in cell culture	Regulation	Function of gene product
$\gamma_1 34.5$	ICP34.5	Y	γ_1	Null mutants are attenuated and fail to block phosphorylation of eIF-2α by activated protein kinase RNA-dependent kinase; carboxyl terminus homologous to the corresponding domain of the GADD34 proteins.
ORF-P	ORF-P	Y	pre α	ORF is antisense to the $\gamma_1 34.5$ gene and repressed by binding of ICP4 to cap site. Proteins interact with p32, a component of SF2/ASF splicing factor.
ORF-O	ORF-O	Y	pre α	Overlaps with ORF P, a protein made by frameshift from ORF-P.
α0	ICP0	Y	α	Promiscuous transactivator, requires ICP4 for optimal activity; nucleotidylylated, phosphorylated by U_L13, nuclear (early) and cytoplasmic (late) phases. Null mutants debilitated at low multiplicities of infection.
U_L1	gL	N	γ	Complex with gH required for transport of both proteins to plasma membrane and for viral entry mediated by gH.
U_L2		Y	β	Uracil DNA glycosylase.
U_L3		Y	γ_2	Nuclear phosphoprotein of unknown function. Reported to localize to perinuclear region early and to the nucleus late in infection.
U_L4		Y	Unknown	Unknown.
U_L5		N	β	Forms complex with U_L8 and U_L52 proteins.
U_L6		N	Unknown	Virion protein; required for DNA cleavage and packaging.
U_L7		N	Unknown	Unknown.
U_L8		N	β	Forms complex with U_L5 and U_L52 (helicase/primase complex). Stabilizes interaction between primers and DNA template.
U_L9		N	γ(?)	Binds to origins of DNA synthesis in sequence-specific (origin) fashion; carries out helicase and ATPase activities.
U_L10	gM	Y	γ	Glycoprotein present in virions and plasma membranes.
$U_L10.5$		?	Unknown	Unknown.
U_L11		Y	γ(?)	Myristoylated protein; necessary for efficient capsid envelopment and exocytosis.
U_L12		Y	β	Exonuclease (DNase) involved in viral nucleic acid metabolism; reported to localize in nucleoli and in virally induced nuclear dense bodies and to bind to *a* sequence along with other unidentified proteins. Complex may be involved in cleavage/packaging of viral DNA.
$U_L12.5$		Y	Unknown	Nuclease-associated with capsids.
U_L13		Y	γ	Virion (nuclear) protein kinase; substrates include ICP0, ICP22, *vhs*, U_L3, U_L49, etc.
U_L14		N	Unknown	Unknown.
U_L15		N	γ	*ts* mutant DNA+. Two exons; protein required for cleavage/packaging of DNA.
U_L16		Y	Unknown	Virion protein; gene located within intron of *U_L15*.
U_L17		N	γ	Located within intron of *U_L15*.
U_L18	VP23	N	γ	Protein required for capsid formation and cleavage/packaging of DNA.
U_L19	VP5, ICP5	N	γ_1	Major capsid protein.
U_L20		Y	γ	Membrane protein, associates with nuclear membranes, Golgi stacks, etc. Essential for viral exocytosis.
$U_L20.5$			γ_2	Unknown.
U_L21		Y	Unknown	Nucleotidylylated phosphoprotein; unknown function.
U_L22	gH	N	γ_2	Forms complex with gL (see above). Required for entry, egress, and cell–cell spread.
U_L23	ICP36	Y	β	Thymidine (nucleoside) kinase.
U_L24		Y	γ	Syn$^-$ locus; membrane-associated protein?
U_L25		N	γ	Virion protein reported to be required for packaging of cleaved viral DNA.
U_L26		N	γ	Serine protease; substrates are U_L26 protein and $U_L26.5$ (IC35). VP21 (C portion of U_L26), VP24 (N terminus of protease) are products of the self-cleavage of U_L26.
$U_L26.5$	ICP35	N	γ	Substrate of U_L26 protease unique to B capsids and forms inner core or scaffolding; the precursor, ICP35b,c is cleaved to c, I. On packaging of DNA it is removed from capsid shell.
U_L27	gB, VP7	N	γ_1	Glycoprotein required for viral entry; forms a dimer and induces neutralizing antibody. A *syn*$^-$ locus maps to the carboxyl terminus.
$U_L27.5$		?	Unknown	Unknown, antisense to gB.
U_L28	ICP18.5	N	γ	M_r 87–95 K protein required for DNA cleavage/packaging.
U_L29	ICP8	N	β	Binds single-stranded DNA cooperatively, required for viral DNA replication: forms complex with DNA polymerase and U_L42. *ts* mutants are DNA$^-$ and hence expression of early and late genes may be affected positively or negatively by ICP8. Because ICP8 denatures DNA, it affects renaturation of complementary strands of DNA and affects homologous pairing and strand transfer.
U_L30		N	β	DNA polymerase; forms complex with ICP8 and C terminal 247 amino acids of U_L42.
U_L31		N	γ_2	Nucleotidylylated phosphoprotein, cofractionates with nuclear matrix.
U_L32		N	γ_2	Cytoplasmic/nuclear protein required for DNA cleavage/packaging.
U_L33		N	Unknown	DNA packaging; necessary for assembly of capsids containing DNA.
U_L34		N	Unknown	Abundant nonglycosylated, membrane-associated, virion protein phosphorylated by U_S3.
U_L35	VP26	N	γ_2	Basic phosphorylated capsid protein.
U_L36	ICP1-2	N	γ_2	Tegument phosphoprotein. DNA is not released from capsids at nuclear pores in cells infected with *ts* mutant. Reported to form complex with a M_r 140 K protein that binds *a* sequence DNA.

Table 1. (Continued)

Gene	Product	Dispensable in cell culture	Regulation	Function of gene product
U_L37	ICP32	N	γ	Cytoplasmic phosphoprotein; in presence of ICP8 it is transported to nucleus and associates with DNA, but phosphorylation is not dependent on ICP8. Required for maturation of virions.
U_L38	VP19C	N	γ_2	Capsid assembly protein, binds DNA and may be involved in anchoring DNA in the capsid.
U_L39	ICP6	Y	β	Large subunit of ribonucleotide reductase. Autophosphorylates via unique N terminus but does not trans-phosphorylate.
U_L40		Y	β	Small subunit of ribonucleotide reductase.
U_L41	VHS	Y	γ	Causes nonspecific degradation of mRNA after infection; shuts off host protein synthesis, enables sequential synthesis of viral proteins.
U_L42		N	β	Double-stranded DNA-binding protein, binds to and increases processivity of DNA polymerase.
U_L43		Y	Unknown	Amino acid sequence predicts membrane-associated protein.
U_L43.5		Y		Antisense to U_L43; low abundance nuclear protein; accumulates in assemblons.
U_L44	gC, VP7.5	Y	γ_2	Glycoprotein involved in cell attachment; required for attachment to the apical surface of polarized MDCK cells.
U_L45		Y	γ_2	Encodes a M_r 18 K protein of unknown function.
U_L46	VP11/12	Y	γ	Tegument phosphoprotein reported to modulate the activity of U_L48 (αTIF).
U_L47	VP13/14	Y	γ_2	Nucleotidylylated tegument phosphoprotein modulates the activity of U_L48 (αTIF).
U_L48	VP16, ICP25, αTIF	N	γ	Tegument protein, induces α genes by interacting with *Oct*I. The complex binds to specific sequences with the consensus GyATGnTAATGArATTCyTTGnGGG-NC.
U_L49	VP22	N	γ	Nucleotidylylated, mono(ADP-ribosyl)ated tegument phosphoprotein.
U_L49.5		N	γ_2	Sequence predicts a M_r 12,000 membrane-associated protein.
U_L50		Y	β	dUTPase.
U_L51		Y	γ	Unknown.
U_L52		N	β	Component of the helicase/primase complex.
U_L53	gK	Y	γ	Glycoprotein required for efficient viral exocytosis; contains *syn*$^-$ locus.
α27	ICP27	N	α	Nucleotidylylated multifunctional regulatory protein; causes redistribution of snRNPs, inhibits RNA splicing. It is required for late gene expression, and negatively regulates early genes.
U_L55		Y	Unknown	Unknown.
U_L56		Y	Unknown	Nuclear, virion-associated protein of unknown function.
α4	ICP4	N	α	Nucleotidylylated, poly(ADP-ribosyl)ated phosphoprotein; regulates positively most β and γ genes and negatively itself, ORF-P and the α0 gene; blocks apoptosis. Binds to DNA in sequence specific fashion.
α22	ICP22	Y	α	Nucleotidylylated regulatory protein, phosphorylated by U_L13 and U_S3 protein kinases, required for optimal expression of ICP0 and of a subset of γ proteins.
U_S1.5	U_S1.5	Y	α	Regulatory protein; extent to which it shares function with ICP22 not known.
U_S2		Y	Unknown	Unknown.
U_S3		Y	β	Protein kinase; major substrate is U_L34 protein.
U_S4	gG	Y	γ	Glycoprotein involved in entry, egress, and spread from cell to cell.
U_S5	gJ(?)	Y	Unknown	Sequence predicts glycoprotein.
U_S6	gD VP17/18	N	γ_1	Glycoprotein required for post-attachment entry of virus into cells.
U_S7	gI	Y	γ	gI and gE glycoproteins form complex for transport to plasma membrane and also to constitute a high-affinity Fc receptor. gI is required for basolateral spread of virus in polarized cells.
U_S8	gE	Y	γ_2	FC receptor; involved in basolateral spread of virus in polarized cells.
U_S8.5		Y	β or γ_1	Unknown.
U_S9		Y	Unknown	Tegument protein phosphorylated by U_L13.
U_S10		Y	Unknown	Tegument protein.
U_S11		Y	γ_2	Tegument protein binds to U_L34 mRNA in sequence- and conformation-specific fashion; binds to the 60S ribosomal subunit and localizes in the nucleolus.
α47	ICP47	Y	α	Binds to TAP1/TAP2 and to block antigen presentation to CD8$^+$ cells.
Ori$_S$TU	Ori$_S$RNA	Y	γ_2	RNA transcribed across S origins of DNA synthesis. Function is not known.
LATU	LATs	Y	pre α?	Transcripts, found in latently infected nerons. Function is not known.

Ori$_S$TU is the transcriptional unit across the origin of DNA synthesis in the S component. LATTU is the transcriptional unit expressed in latently infected sensory neurons. This table was updated and modified from Ref. 1. Additional references are as follows: U_L10.5, ref. 7; U_L12.5, ref. 15; U_L20.5, P. J. Ward and B.R. (unpublished data); U_L27.5, Y. Chang, G. Campadelli-Fiume, and B.R. (unpublished work); U_L43.5, ref. 14; U_S1.5, ref. 9; ORF-O, G. Randall and B.R. (unpublished work); U_S9, R. Brandimasti and B.R. (unpublished work).

terminus of the genome next to the c sequence and contains signals for cleavage of unit length DNA from concatemers and packaging of the DNA in preformed capsids (reviewed in ref. 1). HSV-1 is known to express at least 84 different polypeptides whose open reading frames (ORFs) are distributed as indicated in Fig. 1 (refs. 4 and 7–15; P. L. Ward and B.R., unpublished data; Y. Chang, G. Campadelli-Fiume, and B.R., unpublished data). Of this number, five ORFs, mapping in the inverted repeats, are present in two copies per viral genome. In addition to the ORFs listed in Fig. 1, infected cells contain

transcripts from genome domains not known to specify proteins. These include the LATs discussed below and an RNA (Ori$_S$RNA) derived by transcription of the two of the three origins of viral DNA synthesis mapping in inverted repeats (16, 17). The ORFs form several groups whose expression is coordinately regulated in a cascade fashion. The α genes are expressed first, functional α proteins are required for the expression of β genes, and both functional α proteins and viral DNA synthesis mediated by β proteins are required for (γ_2), or enhance (γ_1), the expression of late or γ genes (18, 19). Whereas α proteins perform regulatory functions or prevent a host response to infection, the function of β proteins is the management of the nucleic acid metabolism and viral DNA synthesis in the infected cell, as well as posttranslational modification of proteins made earlier and later in infection. The γ proteins are largely the structural components of the virions (reviewed in ref 1).

Since 1982 (20, 21) techniques have been available to delete or insert DNA sequences at specific sites. These studies have revealed the existence of ORFs that are expressed and the rather unexpected finding that 45 of the 83 ORFs specifying diverse proteins are dispensable for viral replication in at least some cells in culture. A list of the ORFs and the functions expressed by the gene products are shown in Table 1. The 38 ORFs that cannot be deleted without ablating the capacity of the virus to replicate include four genes specifying surface glycoproteins, two regulatory proteins [infected cell proteins no. 4 (ICP4) and no. 27 (ICP27)], seven proteins required for the synthesis of viral DNA, proteins required for assembly of the capsid, structural proteins, and proteins whose functions are not yet known. The 45 accessory ORFs, which are not required for viral replication in cells in culture, specify 11 proteins involved in entry, sorting, and exocytosis of virus (glycoproteins C, E, G, I, J, K, M; membrane proteins U$_L$11, U$_L$20, U$_L$24, U$_L$43), 2 protein kinases (U$_L$13, U$_S$3), 2 proteins that preclude host response to infection (α47 and $\gamma_1$34.5), 3 regulatory proteins (α0, α22, U$_S$1.5), 5 proteins that augment the nucleotide triphosphate pool or repair DNA (thymidine kinase, dUTPase, ribonucleotide reductase, DNase, uracil glycosylase), 1 protein that causes the degradation of mRNA after infection (U$_L$41), and numerous other proteins whose functions are not known (detailed references in ref. 1).

The Role of Selected Viral Genes in Viral Replication

The reproductive cycle of HSV has been described in detail elsewhere (1). The objective of viral replication is efficient, rapid synthesis and dissemination of viral progeny. In the process, the infected cell dies. Viral replication consists of a series of events very tightly regulated both positively and negatively. To accomplish its objectives, the virus brings into the newly infected cell several proteins packaged in the virion tegument (a layer of proteins located between the capsid and the envelope; see ref. 22), whose functions are best described as creating the environment for initiation of viral replication). One, designated as VP16 or α gene trans-inducing factor (αTIF) induces the transcription of α genes by cellular RNA pol II and accessory factors, whereas another encoded by U$_L$41 causes the degradation of cytoplasmic RNAs (23–25). The major regulatory protein ICP4 made after infection acts both negatively by binding to high-affinity sites on viral DNA and positively by an as yet unknown mechanism (reviewed in ref. 1). The hypothesis that ICP4 is directly involved in transcription is based on reports that it binds TATA box-binding protein and transcription factor IIB, and on the evidence that after the onset of DNA synthesis, it is a component of γ-transcriptons—nuclear stuctures containing newly synthesized viral DNA, RNA polymerase II, ICP22, and a cellular protein known as L22 or EAP and that is normally present in nucleoli and ribosomes and binds small RNA molecules (ref. 26; R. Leopardi, P. L. Ward, W. Ogle, and B.R., unpublished work). ICP27 has multiple functions, but primarily it regulates posttranscriptional processing of RNA (27). ICP22 also appears to be a transcriptional factor; it is required for the expression of the α0 gene and also of a subset of γ genes and is a component of the γ-transcriptons (ref. 28; R. Leopardi, P. L. Ward, W. Ogle, and B.R., unpublished work). Among other proteins that regulate the replicative cycle is U$_L$13, a protein kinase known to mediate the phosphorylation of ICP0, ICP22, and other proteins (ref. 28; W. Ogle, K. Carter, and B.R., unpublished work). The function of another viral protein kinase, U$_S$3, is less clear (1).

The functions of $\gamma_1$34.5 and α47 genes are of particular interest. $\gamma_1$34.5 appears to have at least two functions. One function of $\gamma_1$34.5 is to preclude the shutoff of protein synthesis caused by activation of the protein kinase RNA-dependent kinase and, ultimately, by the phosphorylation of the α subunit of the translation initiation factor eIF-2 (29). The carboxyl-terminal domain of $\gamma_1$34.5 required for this function is homologous to the corresponding domain of the mammalian protein GADD34—one of a set of proteins induced in growth arrest as a consequence of differentiation, serum deprivation, or DNA damage. Human GADD-34, or a chimeric gene consisting of the amino terminal domain of $\gamma_1$34.5 and the carboxyl terminus of GADD-34, effectively replaces the $\gamma_1$34.5 gene in the context of the viral genome (30). The second function of the $\gamma_1$34.5 enables the virus to multiply efficiently in a number of tissues, but particularly in the CNS of experimental animal systems (31, 32). The argument that this function of $\gamma_1$34.5 is independent of the function of the protein to preclude the phosphorylation of eIF-2α is based on the observation that viruses carrying GADD-34 in place of $\gamma_1$34.5 are not blocked in protein synthesis; they are nevertheless attenuated (32).

α47 binds the complex of TAP1/2 and thereby precludes the transport of peptides for presentation to CD8$^+$ cells (33).

The Function of Viral Genes in Latency

To date the only domain of the viral genome shown to be expressed during latency maps in the inverted repeats flanking U$_L$ (16). The RNAs described to date consist of two populations. The low abundance population arises from an 8.3-kbp domain. The two abundant RNAs, of 2 and 1.5 kb respectively, and known by the acronym LAT, appear to be stable introns that accumulate in abundant amounts in nuclei of neurons harboring latent virus. Deletion of the upstream promoter or of the sequences encoding LATs has little effect on the establishment or maintenance of the latent state, but reduces the efficiency of latent virus to reactivate (1). LATs may be harbingers of neurons capable of reactivating than viral products required for establishment of latency. Given the multitude of viral accessory genes whose function is to render viral replication and dissemination more efficient, the notion that the virus depends solely on the cellular factors for dissemination seems unlikely. Recent studies have shown that the genome domain transcribed during latency contains ORF-0 and ORF-P, whose expression is repressed by ICP4, inasmuch as mutagenesis of the high-affinity binding site at the transcription initiation site of ORF-P led to the derepression of both genes (12). The virus carrying the derepressed gene is attenuated in experimental animal systems (mice) and underexpresses α0 and α22 proteins (34, 45). In addition, ORF-P protein colocalizes and binds to a protein (p34), which is a component of the SF2/ASF splicing factor (45). The role of ORF-P in latency is not known.

Genetic Engineering of Novel Viral Genomes

The two major techniques for construction of novel viruses depend on genetic recombination in infected or transfected cells. The first technique was based on the observation that transfection of cells with intact viral DNA and mutated fragment will result in a small fraction of the progeny carrying the mutated sequence (20, 21). To specifically select this population, the procedure first involved the insertion at or near the target for deletion by recombination through flanking sequences of the viral thymidine kinase as a selectable marker. Only the progeny of transfection, which carries the viral thymidine kinase gene, would multiply in thymidine kinase minus cells overlaid with appropriate medium. In the second step, the inserted thymidine kinase was deleted along with adjacent target sequences by recombination through flanking sequences with a mutated DNA sequence. In this instance, only thymidine kinase minus progeny would break through efficiently in cells overlaid with medium containing bromouracil deoxyriboside. A minor variant of this technique based on gene inactivation by random insertion of the mini-μu phage has the disadvantage in that the mini-μu DNA sequence is quite large and troublesome to remove (21).

The second procedure involves transfection of cells with overlapping cosmids containing appropriate insertions or deletions. Expression of genes contained in cosmids leads eventually through recombination to the reconstruction of full-length viral genomes. This procedure is less efficient, but the progeny of transfection need not be subjected to selection for the isolation of the desired genotype (35)

Both procedures suffer from gene rearrangements as a result of transfection. To link a specific genotype to the observed phenotype, it is essential to determine whether the wild-type phenotype is restored by the repair of the deleted sequences with a small DNA fragment. It is estimated that as much as 30% of the recombinants made by the techniques described above contain additional mutations detected only after the restoration of the missing sequence.

Requirements and Design of Viral Vectors

Vectors for delivery of cellular genes to CNS must not express viral genes that cause the infected cell to make cytotoxic viral proteins or that induce an immune response, which may damage the recipient cells. A huge literature describes attempts to obtain long-term expression of reporter genes in experimental animals, particularly mice. A potentially suitable vector for this type of application is based on construction of defective genomes, i.e., genomes that are unable to replicate in the recipient cells. In recent years, two different types of defective recombinant viruses have emerged. The first is based on defective HSV genomes, which arise spontaneously by recombination and are amplified during serial passage at high multiplicities (36). The defective genome subunit (the amplicon) consists minimally of the terminal a sequence and an origin of viral DNA synthesis. In virions, these unit are arranged head-to-tail. HSV-1 amplicons have long been shown to express efficiently cellular genes incorporated into them (37). Amplicons do not encode viral proteins and must therefore be supplied with both viral structural proteins and proteins required for viral DNA synthesis and exocytosis in order to be made. In theory, amplicons could accommodate as much as 150 kbp of DNA. In practice, three problems exist. First, until recently, amplicons were contaminated with helper DNA. Second, the amounts of amplicons made are not readily controllable and the usual yields are several orders of magnitude lower than those of wild-type infectious virus. Third, amplicons tend to be unstable on serial passage because the smaller the amplicon, the greater is its selective advantage (38). Of these problems, only the first one has been solved, since a helper virus incapable of packaging has been constructed (39). A significant potential problem is the rescue of the helper virus by recombination with the amplicon, which would enable the helper to package.

The second approach is based on construction of viruses lacking essential genes. The essential genes deleted singly or in combination include α4, α22, α27, U_L48 (α gene trans-inducing factor or VP16), and U_L41 (40). Deletion of the inverted repeats (b'a'c') and of stretches of genes not essential for viral replication in cell culture (e.g., U_S except for U_S6, α22, and α47), and large stretches of U_L (U_L2, U_L3, U_L4, U_L10, U_L11, U_L43, U_L43.5, U_L45, U_L46, U_L47, U_L55, U_L56) could make space for insertion of at least 40 kbp of DNA. By necessity, the debilitated recombinant virus must be grown in cell lines expressing the deleted viral genes. This type of vector also presents several problems. Foremost is that some of the selected genes targeted for deletion perform a multiplicity of functions. For example, they may be both transactivators and repressors, and they normally block the cells from programmed cell death triggered by viral gene expression (41). It could be predicted therefore that sooner or later the recipient cell will cease functioning because of slow but ultimately fatal expression of viral genes. Other problems, perhaps more readily surmountable, include potential for recombination between the defective viral genomes and viral genes resident in the cell genome and the stability of the cell lines. In neither model is reactivation from latency a problem inasmuch as the viruses are unable to replicate.

Whereas gene therapy may require delivery of therapeutic genes to a significant fraction of cells that normally express them or could serve as surrogate expressors, cancer therapy requires complete destruction of cancer cells. Theoretically, for selective destruction of cancer cells, it should be sufficient to introduce a defective viral genome expressing a factor that is excreted in abundant amounts and toxic only for cancer cells. Theory not withstanding, viruses that infect or at least selectively multiply and destroy tumor cells are likely to have a therapeutic advantage. In experimental animal systems several genetically engineered viruses appear to have met at least the initial requirements for further development (32). The mutations that render the viruses attenuated fall into three categories. The first set encodes enzymes (e.g., thymidine kinase, ribonucleotide reductase, etc.) involved in nucleic acid metabolism and that would not be available in neurons but would be available to the virus in dividing tumor cells. The second category are genes encoding proteins (e.g., $\gamma_1$34.5), which disable the capacity of the virus to replicate in CNS for reasons not well understood, as described earlier in the text (reviewed in ref. 32). Lastly, deletion of inverted repeats (b'a'c') in itself grossly debilitates HSV-1 (42, 43). Irrespective of the set of genes involved for attenuation, it will be virtually impossible to introduce virus in all cancer cells. As in the case of defective viruses, even replicating viruses will have to carry and express factors that induce immune response or activate pro-drugs selectively in cancer cells.

The use of recombinant viruses for prophylaxis against viral infection requires mutants that are genetically stable, incapable of replicating in CNS, incapable of spreading in immunocompromised individuals, unable to reactivate, not transmissible from immunized individual to contacts, but immunogenic and protective against disease caused by subsequent infection. A large number of defective viruses have been proposed for immunization on the grounds that although they cannot produce infectious progeny and fundamentally make proteins only in the set of initially infected cells, the presentation to the immune system of viral antigens made in these cells is superior to that of subunit vaccines (e.g. ref. 44). The central issue is the immunogenic mass required for effective immunity and whether such a mass could be achieved by progeny of a replication-defective virus. Secondary issues are the stability of

the cell lines expressing the complementing viral genes, rescue of the defective virus by recombination, and qualification of transformed cell lines for administration of virus along with cellular DNA to healthy individuals. The construction of replicating virus is not less daunting, and parallels in many respects the construction of recombinant viruses for cancer therapy.

Conclusions

Development of genetic engineering evolved *pari passu* with our knowledge of viral gene content and function and is at a point where, subject to constraints imposed by the size of the virion, the construction of virtually any vector is feasible. The outstanding issues are not the construction of viral vectors, but rather (*i*) delivery of recombinant viruses to appropriate cells, (*ii*) regulation of expression of the gene carried by the viral vector, and (*iii*) regulatory issues related to qualification of continuous cell lines, which express complementing viral genes for replication of defective vectors. Theoretically, it should be possible to create viruses that carry surface protein destined for receptors present only on a specific set of cells, but this is not yet feasible. The problems associated with regulation of gene expression are particularly vexing because the available size for packaging genes in the viral genome is too small to accommodate the genomic versions rather than the cDNA version of most cellular genes of interest. As a consequence, alternative splicing to produce isoforms of cellular proteins and natural regulation of gene expression may not be feasible. The third issue arises from the difficulty of purifying virus away from cell debris which contains cellular DNA. Since only continuous cell lines are likely to express stably viral genes complementing replication defective viruses, it would be necessary to define the requirements for the qualification of such cell lines for production of viruses intended for human use. Considering the progress of the past 10 years, the solution of these problems should not be far behind.

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An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma

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ABSTRACT **Viruses used for gene therapy are usually genetically modified to deliver therapeutic transgenes and prevent viral replication. In contrast, replication-competent viruses may be used for cancer therapy because replication of some viruses within cancer cells can result in their destruction (oncolysis). Viral ribonucleotide reductase expression is defective in the HSV1 mutant hrR3. Cellular ribonucleotide reductase, which is scarce in normal liver and abundant in liver metastases, can substitute for its viral counterpart to allow hrR3 replication in infected cells. Two or three log orders more of hrR3 virions are produced from infection of colon carcinoma cells than from infection of normal hepatocytes in viral replication assays. This viral replication is oncolytic. A single intravascular administration of hrR3 into immune-competent mice bearing diffuse liver metastases dramatically reduces tumor burden. hrR3-mediated tumor inhibition is equivalent in immune-competent and immune-incompetent mice, suggesting that viral oncolysis and not the host immune response is the primary mechanism of tumor destruction. HSV1-mediated oncolysis of diffuse liver metastases is effective in mice preimmunized against HSV1. These results indicate that replication-competent HSV1 mutants hold significant promise as cancer therapeutic agents. Yoon, S. S., Nakamura, H., Carroll, N. M., Bode, B. P., Chiocca, E. A., Tanabe, K. K. An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma. *FASEB J.* 14, 301–311 (2000)**

Key Words: HSV1 · gene therapy · ribonucleotide reductase

AS A RESULT of evolutionary forces, viruses have necessarily evolved efficient mechanisms to deliver their genetic material into cells, avoid cellular defenses, and induce host cells to transcribe and translate viral genes. Accordingly, several viruses have been adapted for delivery of therapeutic genes to both normal cells and cancer cells, including retrovirus, adenovirus, adeno-associated virus, vaccinia virus, and herpes simplex virus type 1 (HSV1) (1, 2). Most gene therapy research has been devoted to the development of strategies that allow viruses to deliver their genetic payload without subsequent viral replication. It has been a long-held belief that viruses used for delivery of genes into human cells for therapeutic purposes should be rendered incapable of replication based on concerns that viral replication may lead to cellular transformation or produce significant illness. The overwhelming majority of cancer gene therapy strategies reported to date use replication-incompetent viruses (3). These viruses have served principally as vehicles for delivery of therapeutic genes, such as cytokines, suicide genes, costimulatory molecules, or tumor suppressor genes. However, viruses engineered to remain replication-competent may be exploited for cancer therapy because viral replication within cancer cells results in oncolysis and produces progeny virion that can infect adjacent cancer cells.

There has been growing interest in oncolytic viruses, which have recently been introduced into clinical trials (4). One particular replication-competent adenovirus that has received considerable attention, Onyx-015, is defective in viral E1B 55 kDa protein expression (5). It has been proposed that the principal mechanism of tumor destruction by Onyx-015 results from adenoviral replication specifically within p53-deficient cells, although this concept has recently been challenged (6–8). Reovirus has also been examined as a replication-competent oncolytic virus (9) because of its preferential replication in cells with an activated *ras* pathway (10).

HSV1 is a double-stranded DNA virus that has been adapted for cancer therapy. HSV1-based vectors were initially examined as potential vehicles for gene transfer into the central nervous system. Un-

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wanted cytopathic effects resulting from viral infection plagued these early experiments (11, 12). However, these cytopathic effects have subsequently been exploited to treat cancer. Entry of wild-type HSV1 into cancer cells leads to a sequential cascade of viral gene expression that ultimately results in production of multiple progeny virions and cell death (13). Progeny virion can then infect adjacent cancer cells to enhance the anti-tumor effects. Unlike most cancer gene therapy strategies, those that rely on oncolysis induced by viral replication do not require prolonged transgene expression. HSV1 mutants have been engineered that are defective in expression of genes that are important but not essential for viral replication, such as thymidine kinase (14), ribonucleotide reductase (15), and gamma 1 34.5 (16). These mutants appear to replicate more robustly in tumor cells than in normal cells, and have been directly inoculated into brain tumors in animals to achieve anti-tumor effects (14, 16, 17). Unfortunately, treatment strategies that require direct viral inoculation into each and every tumor nodule are neither feasible nor effective for treatment of solid tumor metastases, which typically present as multiple and diffuse tumor nodules. Accordingly, we have examined a cancer therapy approach designed to target an HSV1 mutant for diffuse liver metastases after intravascular delivery. Here we show that an oncolytic HSV1 mutant defective in ribonucleotide reductase expression replicates preferentially in tumors and not in surrounding normal liver after intravascular delivery into mice bearing diffuse liver metastases. Moreover, this viral replication produces significant anti-neoplastic effects after a single intravascular administration, whereas replication-incompetent HSV1 mutants do not produce any measurable anti-tumor activity against liver metastases. Inhibition of tumor growth is equivalent in immune-competent and immune-incompetent mice. We also demonstrate that HSV1-mediated oncolysis of diffuse liver metastases is effective in mice preimmunized against HSV1.

MATERIALS AND METHODS

Cells and viruses

HT29 human colon carcinoma cells and Vero African Green Monkey kidney cells were obtained from the American Type Culture Collection (Rockville, Md.). MC26 mouse colon carcinoma cells were obtained from the National Cancer Institute Tumor Repository (Frederick, Md.). E5 cells (Vero cells stably transfected with ICP4) and V27 cells (Vero cells stably transfected with ICP27) were kindly provided by D. M. Knipe (Harvard Medical School). HT29 and MC26 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 1:1 Hamm's F-12 supplement, 8% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Vero, E5, and V27 cells were maintained in DMEM with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. E5 and V27 cells were cultured in the presence of 400 μg/ml G418. Primary human hepatocytes were prepared from fresh human liver specimens obtained from the operating room as described (18, 19). Primary mouse hepatocytes were prepared from BALB/c mouse fresh liver specimens in a similar manner. Primary hepatocytes were maintained in William's medium E containing bovine serum albumin, insulin, transferrin, selenium, trace elements, dexamethasone, linoleic acid, linolenic acid, glucagon, penicillin, streptomycin, and fungizone on collagen-coated plates. The HSV 1 vectors hrR3 (15) (kindly provided by S. K. Weller, University of Connecticut), *d*120 (20), and *d*27 (21) (both kindly provided by D. M. Knipe, Harvard Medical School) were derived from the parental wild-type strain KOS (kindly provided by D. M. Coen, Harvard Medical School). hrR3 and KOS were propagated and titered on Vero cells, and *d*120 and *d*27 were propagated and titered on E5 and V27 cells, respectively. Heat inactivation of hrR3 was performed as described (22).

Viral replication and cytotoxicity assays

Viral replication assays were performed as described (21). Briefly, 3×10^6 cells were infected with 6×10^5 plaque forming units (pfu) of virus for 2 h, at which time unadsorbed virus was removed by washing with a glycine-saline solution (pH 3.0). Forty hours after infection the supernatant and cells were harvested, exposed to three freeze/thaw cycles to release virions, and titered on Vero cells. The results are the combination of three independent experiments. Viral cytotoxicity assays were performed as described (23). Briefly, cells were plated onto 96-well plates at 5000 cells per well for 36 h. Virus was added at multiplicity of infection (moi) values ranging from 0.0001 to 10 and incubated for 6 days. The number of surviving cells was quantitated using a colorimetric MTT assay. Tests were performed in quadruplicate.

Western blot analysis

Cell and liver tissue lysates containing equal amounts of protein as determined by BCA protein assay reagent (Pierce, Rockford, Ill.) were resolved by gel electrophoresis on 4–20% Tris-glycine polyacrylamide gels (Novex, San Diego, Calif.), and proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Membranes were blocked with 5% nonfat milk overnight at 4°C. Membranes were incubated with 5 μg/ml mouse anti-ribonucleotide reductase mAb (MAB3033, Chemicon International, Inc., Temecula, Calif.) for 1 h or 0.44 μg/ml mouse anti-β-actin mAb (A-5441, Sigma, St. Louis, Mo.) for 1 h. After washing three times, membranes were incubated with peroxidase linked anti-mouse immunoglobulin (NA 931, Amersham Life Sciences, Arlington Heights, Ill.) diluted 1:4000 for 1 h. Specific proteins were detected using an enhanced chemiluminescence (ECL) system following the manufacturer's instructions (Amersham Life Sciences, Inc.).

Animal studies

BALB/c and athymic BALB/c (nu/nu) mice were obtained from Charles River Labs (Wilmington, Mass.). Animal studies were performed in accordance with policies of the Massachusetts General Hospital Subcommittee on Research Animal Care. To examine sites of hrR3 replication in livers, MC26 liver metastases were generated by injection of a single-cell suspension of 1×10^5 MC26 cells in 100 μl HBSS without Ca^{2+} or Mg^{2+} into the spleens of BALB/c mice. Eight days later, 1×10^7 or 1×10^8 pfu hrR3 in 100 μl media was

injected into a non-tumor-bearing portion of the spleen (n=3 per group). The presence of tumor in the spleen does not affect delivery of virus to the liver after inoculation of virus into the spleen (data not shown). Mice were killed 12 days after tumor inoculation.

For partial hepatectomy experiments, mice underwent a 75% hepatectomy or sham laparotomy as described (24). Mice were killed at specified times, and livers were harvested to assay for ribonucleotide reductase expression (n=2 per group). To determine whether hrR3 replicates in regenerating mouse livers, 1×10^8 pfu hrR3 in 100 μl media was injected into the spleen 4 days after partial hepatectomy (n=3 per group). Mice were killed 8 days later.

To assess the therapeutic efficacy of HSV1 against early liver metastases, a single-cell suspension consisting of 1×10^5 MC26 cells in 100 μl HBSS without Ca^{2+} or Mg^{2+} was injected into spleens of BALB/c mice, followed 3 days later by 5×10^7 pfu hrR3, heat-inactivated hrR3, *d*27, *d*120 in 100 μl media or media alone (n=4 per group). Mice were killed 14 days after tumor implantation. To assess therapeutic efficacy against late liver metastases, a single-cell suspension of MC26 cells was injected into the spleen, and mice were then treated with either hrR3 or heat-inactivated hrR3 7 days after tumor implantation (n=5 per group). Again, mice were killed 14 days after tumor implantation.

To examine the therapeutic efficacy of hrR3 in preimmuned mice with liver metastases, 1×10^7 pfu KOS in 100 μl media was injected into the subcutaneous (s.c.) flanks of mice. This had previously been demonstrated to protect mice from subsequent lethal challenge with HSV1 (25). Control mice were vaccinated with media instead. Two mice in each group were killed after 28 days to collect serum for measurement of the presence of antibodies capable of neutralizing hrR3 cytotoxicity against MC26 cells. This cytotoxicity assay was performed as described above, except in this case hrR3 was incubated with one of the four mouse serum samples for 30 min prior to dilution and application to MC26 cells. In a separate experiment, mice preimmunized with either KOS or media were injected with MC26 cells into the spleen after 25 days and then treated 5×10^7 pfu hrR3 into the spleen after 3 more days (n=5 per group). Mice were killed 14 days after tumor implantation.

Subcutaneous tumors were generated by implantation of 2.5×10^6 MC26 cells in 100 μl HBSS s.c. into the flanks of immune-competent BALB/c mice or immune-incompetent athymic BALB/c (nu/nu) mice. Eight and 11 days later, 1×10^8 pfu hrR3 in 50 μl media or media alone was injected into the center of the developing tumor (n=5 per group). Flank tumor length (L) and width (W) were determined every 3–4 days; tumor volume (TV) was determined by the following formula: $TV = (L \times W^2)/2$. In a separate experiment, only 1×10^4 MC26 cells in 100 μl HBSS were injected into the spleens of BALB/c mice, followed 3 days later by either 5×10^7 pfu hrR3 or media alone (n=5 per group). Eight days after intrasplenic tumor inoculation, 2.5×10^6 MC26 cells were implanted s.c. into the flank and tumor volume was determined every 3–4 days. In this experiment, 1×10^4 MC26 cells were initially implanted into the spleen rather than 1×10^5 MC26 cells in order to allow the mice to live long enough to monitor their flank tumor growth.

Histochemistry

Histochemical staining to detect β-galactosidase expression was performed as described (19). Briefly livers were perfused *in vivo* with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS, harvested, soaked in 4% paraformaldehyde in PBS for 2 h, soaked in 30% sucrose in PBS for 24–48 h, and snap frozen in OCT compound in liquid nitrogen. Five micron frozen sections were prepared and incubated in X-gal solution (Fisher Scientific, Pittsburgh, Pa.) overnight. Sections were counterstained with neutral red (Sigma).

Statistical analysis

Mean liver and spleen weights and flank tumor volumes were compared using an unpaired two-tailed *t* test (InStat, Graphpad Software, New York, N.Y.).

RESULTS

An HSV1 mutant replicates more efficiently in colon carcinoma cells than in hepatocytes

Liver metastases express significantly higher levels of ribonucleotide reductase compared to surrounding normal liver (23). To exploit this difference in ribonucleotide reductase expression, we selected an HSV1 mutant, hrR3, that is defective in infected cell protein 6 (ICP6) expression because of an insertion of the *Escherichia coli* β-galactosidase gene into this locus (15). ICP6 serves as the large subunit of viral ribonucleotide reductase. We reasoned that hrR3 replication will proceed more robustly in cells with high levels of cellular ribonuceotide reductase and high intracellular pools of deoxyribonucleotides. To test this hypothesis, we compared replication of hrR3 with that of a wild-type HSV1 strain, KOS, in primary cultures of human hepatocytes and in HT29 human colon carcinoma cells (**Fig. 1*A***). hrR3 replicated nearly as efficiently as KOS in the human colon carcinoma cells; however, as expected hrR3 replication was three log orders less than that of KOS in human hepatocytes. Levels of cellular ribonucleotide reductase were significantly higher in HT29 cells than in human hepatocytes as assessed by Western blot (Fig. 1*A*, inset). We performed a similar analysis in a mouse model, comparing replication of both viruses in primary cultures of mouse hepatocytes and in MC26 mouse colon carcinoma cells (Fig. 1*B*). As was observed in the human tissue system, hrR3 replication was similar to that of KOS in the colon carcinoma cells, but nearly three log orders less than that of KOS in the mouse hepatocytes. HSV1 displays tropism for human and primate cells relative to mouse cells (13), and the overall efficiency of replication observed in the mouse system was less than in the human system. Nonetheless, a significant differential in replication efficiency was still observed. And similar to the human cells, mouse colon carcinoma cells expressed significantly higher levels of ribonucleotide reductase compared to mouse hepatocytes (Fig. 1*B*, inset).

We have previously demonstrated that hrR3 replicates preferentially in human colon carcinoma liver metastases compared to normal liver cells after por-

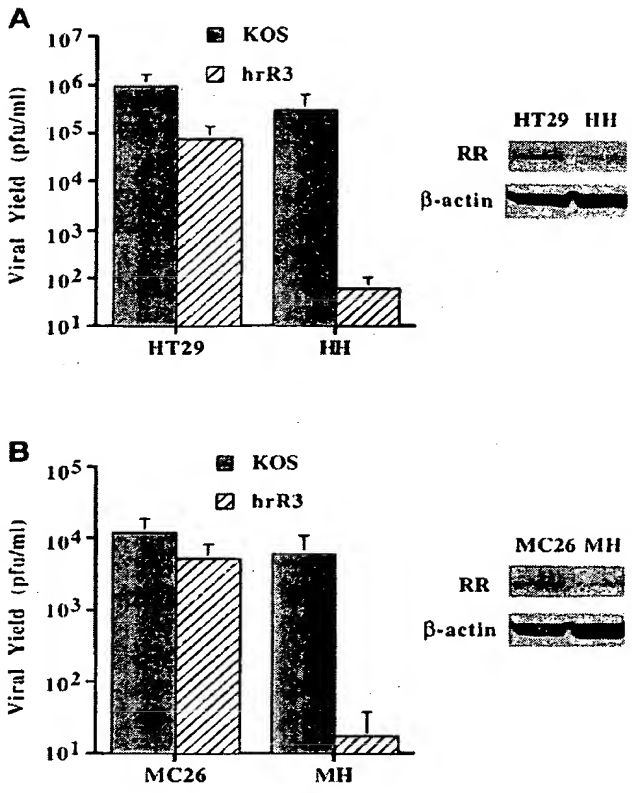


Figure 1. Replication of hrR3 and KOS in colon carcinoma cell lines and primary hepatocytes. *A)* Human colon carcinoma cells (HT29) and human hepatocytes (HH) were infected with hrR3 or KOS using a moi value of 2. Virus recovered from cells and media 40 h later were titered on confluent layers of Vero cells. Ribonucleotide reductase (RR) expression was measured by Western blot analysis and is shown relative to β-actin expression. *B)* Mouse colon carcinoma cells (MC26) and mouse hepatocytes (MH) were similarly examined for HSV yield and RR expression.

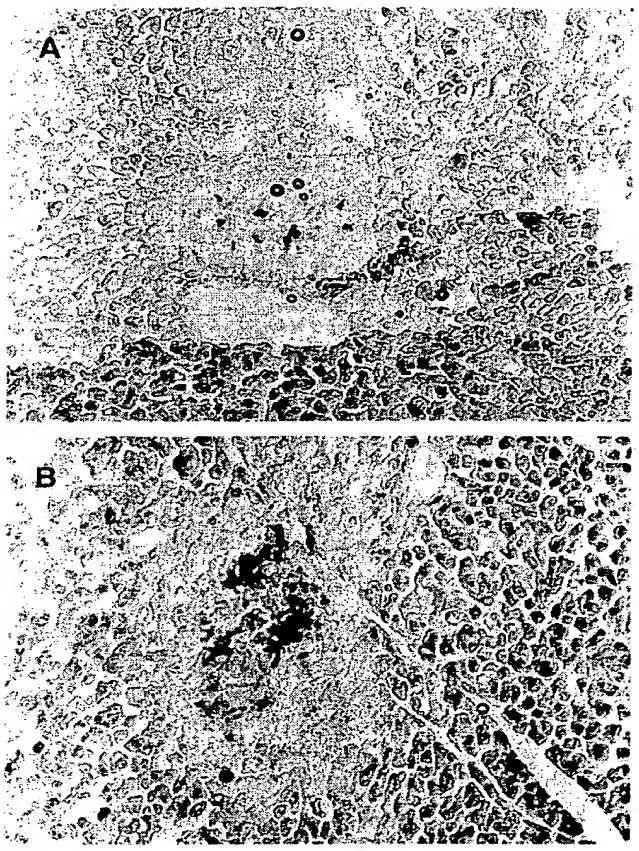


Figure 2. hrR3 selectively replicates in liver metastases. 1×10^6 MC26 cells were injected into the spleens of BALB/c mice, followed 8 days later by intrasplenic injection of 1×10^7 pfu hrR3 (*A*) or 1×10^8 pfu hrR3 (*B*). Four days later livers were harvested, sectioned, and stained for β-galactosidase expression. Representative sections are shown (original magnification = 100×).

tal venous administration in a nude mouse xenograft model (23). However, the tropism of HSV1 for human cells relative to mouse cells made interpretation of these results difficult. Accordingly, we examined hrR3 replication in BALB/c mice bearing syngeneic MC26 colon carcinoma liver metastases. To establish experimental liver metastases, we implanted 1×10^5 MC26 cells into the spleens of BALB/c mice, and 8 days later we inoculated either 1×10^7 or 1×10^8 pfu of hrR3 into the spleen. Four days after inoculation with the lower dose of hrR3, ~50% of tumor nodules stained blue when examined for β-galactosidase expression (**Fig. 2*A***). However, only a minority of cells within any tumor deposit stained blue. After inoculation with the higher dose of hrR3, more than 90% of tumor nodules harbored blue-stained cells, and the overall extent of β-galactosidase expression within each nodule was generally greater (Fig. 2*B*). Notably, the surrounding normal liver parenchyma did not contain any cells that expressed β-galactosidase, including hepatic endothelial cells. We did not observe any β-galactosidase expression in brain or lung specimens either (data not shown).

To confirm that the preferential hrR3 replication in metastatic tumor deposits resulted from the general absence of cell division in the normal liver, we experimentally increased the level of cell division in the normal liver by performing a 75% hepatectomy in BALB/c mice. This procedure has been demonstrated to produce hypertrophy of the remaining liver (24, 26). We confirmed that ribonucleotide reductase levels are low in mouse livers, but quickly rise in response to partial hepatectomy (**Fig. 3*A***). This increase in ribonucleotide reductase levels persists for at least 10 days, and corresponds to hepatic regeneration. In a separate set of experiments, BALB/c mice were subjected to 75% hepatectomy or sham laparotomy; 4 days later, 1×10^8 pfu of hrR3 was inoculated into their spleens. Livers were har-

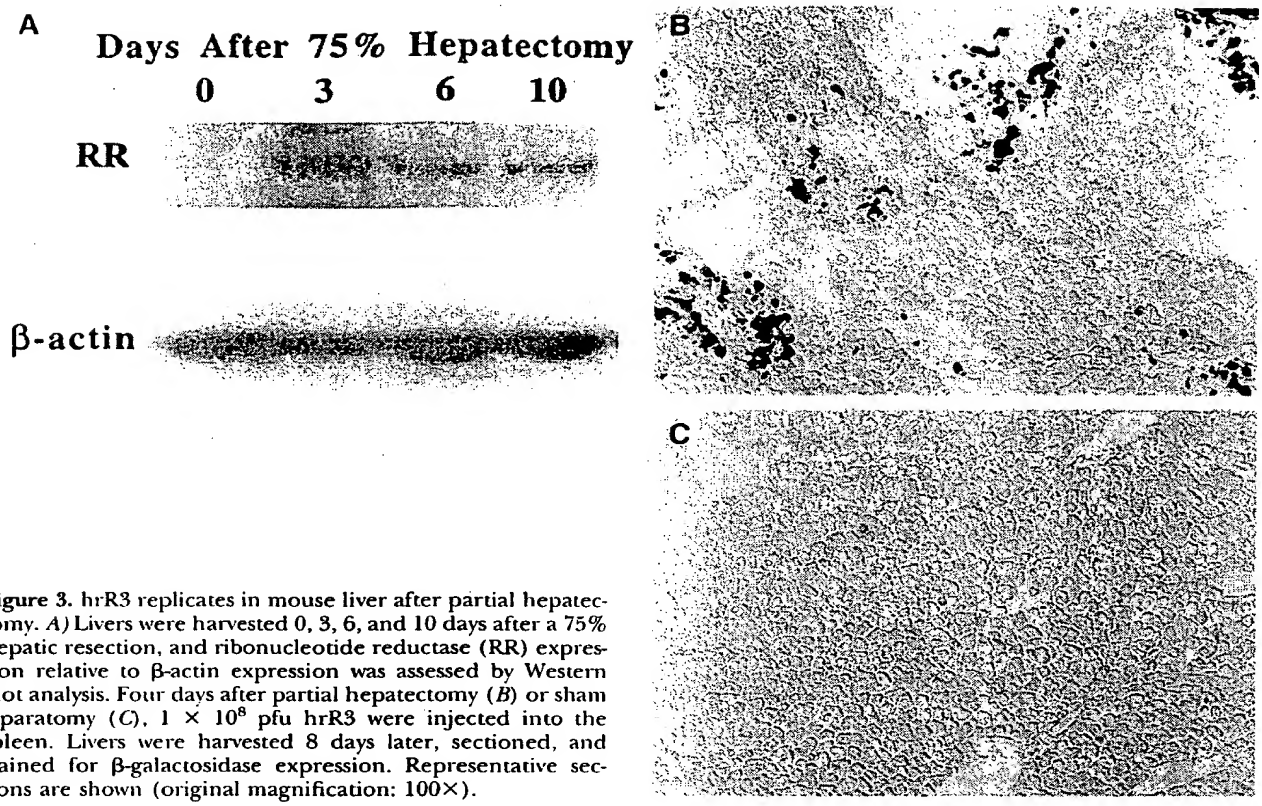


Figure 3. hrR3 replicates in mouse liver after partial hepatectomy. *A)* Livers were harvested 0, 3, 6, and 10 days after a 75% hepatic resection, and ribonucleotide reductase (RR) expression relative to β-actin expression was assessed by Western blot analysis. Four days after partial hepatectomy (*B*) or sham laparatomy (*C*), 1×10^8 pfu hrR3 were injected into the spleen. Livers were harvested 8 days later, sectioned, and stained for β-galactosidase expression. Representative sections are shown (original magnification: 100×).

vested 8 days later and examined for β-galactosidase expression. There were numerous islands of blue-staining cells in the mice subjected to hepatectomy and no blue-staining cells in the livers from the mice subjected to sham laparotomy (Fig. 3*B, C*) or in livers from mice subjected to hepatectomy without subsequent administration of virus (data not shown). The blue-stained cells were generally observed in clusters rather than as individual cells, which suggests that the progeny virions produced from hrR3 replication infected adjacent cells. Thus, hrR3 does not appear to replicate in quiescent liver cells, but does replicate in a regenerating liver.

HSV1 replication produces significant oncolytic effects

Because the β-galactosidase gene in hrR3 is under the control of the promoter for the HSV1 early gene ICP6, expression of this marker gene occurs early in the course of viral infection and is not necessarily associated with completion of the lytic replicative cycle. Accordingly, we directly examined the oncolytic effects associated with infection of tumor cells by replication-conditional and replication-incompetent HSV1 mutants. *d*120 and *d*27 are HSV1 mutants defective in expression of the immediate-early proteins ICP4 and ICP27, and are therefore capable of replication only in cells transformed with ICP4 or ICP27, respectively (20, 21). HT29 human colon carcinoma cells and MC26 murine colon carcinoma cells were infected with hrR3, heat-inactivated hrR3, *d*120, or *d*27 in increasing moi values. HT29 cell destruction was nearly complete 6 days after hrR3 infection when using titers as low as one viral pfu per 100 tumor cells (**Fig. 4***A*). In contrast, very little cytotoxicity was observed with heat-inactivated hrR3, *d*120, or *d*27. The minimal cytotoxicity observed after infection with *d*120 or *d*27 using the highest moi value is probably secondary to HSV1 virus-induced host shutoff protein rather than HSV1 replication (27). As expected, mouse colon carcinoma cells were slightly less susceptible to the cytopathic effects of hrR3 infection (Fig. 4*B*). The moi values that produce nearly complete cell destruction are one to two log orders lower than those required of replication-competent reovirus (9). We observed similar oncolytic effects of hrR3 infection in a panel of several different human and mouse colon carcinoma cell lines (data not shown; ref 19).

Although we observed evidence of hrR3 replication within tumor nodules and not in normal liver after intraportal delivery, the therapeutic efficacy of this replication has never been demonstrated. Therefore, we examined the oncolytic efficacy of hrR3 when administered intravascularly to treat diffuse

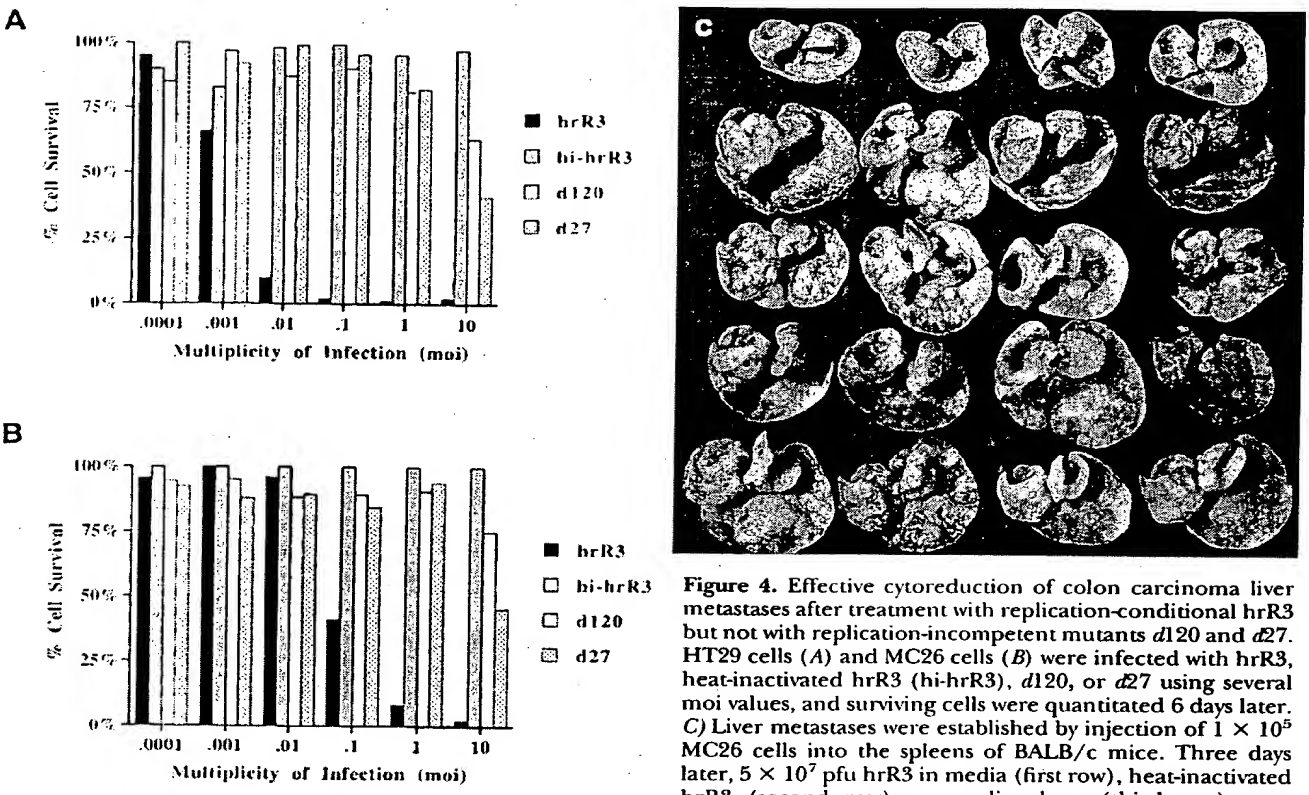


Figure 4. Effective cytoreduction of colon carcinoma liver metastases after treatment with replication-conditional hrR3 but not with replication-incompetent mutants *d*120 and *d*27. HT29 cells (*A*) and MC26 cells (*B*) were infected with hrR3, heat-inactivated hrR3 (hi-hrR3), *d*120, or *d*27 using several moi values, and surviving cells were quantitated 6 days later. *C)* Liver metastases were established by injection of 1×10^5 MC26 cells into the spleens of BALB/c mice. Three days later, 5×10^7 pfu hrR3 in media (first row), heat-inactivated hrR3 (second row), or media alone (third row) were injected into the spleen. Eleven days later, livers and spleens were harvested and analyzed. Mice bearing MC26 liver metastases were similarly treated with HSV1 mutants *d*120 (fourth row) and *d*27 (fifth row) and killed at the same time point.

liver metastases. We used a well-described model of experimental liver metastases involving tumor cell implantation into the spleen (28) rather than the portal vein, because portal vein implantation in BALB/c mice frequently leads to thrombosis and death. Also, we chose to examine the therapeutic efficacy of administration of hrR3 regionally into the portal vein rather than into the tail vein, because we have observed that portal venous inoculation produces significantly higher levels of hrR3 replication in liver metastases compared to tail vein inoculation (data not shown). Diffuse colon carcinoma liver metastases were established in syngeneic BALB/c mice by intrasplenic inoculation of 1×10^5 MC26 tumor cells, and mice were then randomized to treatment with a single intrasplenic inoculation of 5×10^7 pfu hrR3, 5×10^7 pfu heat-inactivated hrR3, or media alone. When mice were killed 14 days after initial tumor cell implantation, all of the mice treated with heat-inactivated hrR3 or media alone had distended abdomens with bloody ascites and some were moribund. In contrast, the hrR3-treated animals all appeared healthy. The number of tumor nodules in the hrR3-treated group ranged from one to five per animal, whereas the nodules in the mice treated with heat-inactivated virus or media alone were too numerous to count (Fig. 4*C* and **Table 1A**).

The striking difference in liver tumor burden that resulted from this single administration of virus also yielded a difference in liver tumor weights that was statistically significant. Two representative livers from each group were sectioned and stained with hematoxylin and eosin. Both hrR3-treated livers contained tumor nodules, although the size of the nodules was markedly smaller than in livers of animals treated with HBSS or heat-inactivated virus. In livers treated with heat-inactivated hrR3 or media, much of the liver parenchyma was replaced by tumor. A significant inflammatory response was not evident in any of the liver sections and no histopathological evidence of hepatotoxicity was observed in areas of normal liver (data not shown).

In this treatment model, virus was inoculated into the spleens just 3 days after tumor cell implantation, when liver metastases are microscopic and not visible by gross inspection. Although the size and weight of splenic tumors did not differ significantly between control mice and hrR3-treated mice, it is possible that much of the anti-tumor effect resulted from hrR3 oncolysis of tumor cells within the spleen prior to their metastasis to the liver. The inability to reproducibly inoculate tumor cells or virus directly into the hepatic artery or portal vein in these mice necessitated a different strategy to address this issue.

TABLE 1. *Liver and splenic tumors following treatment of MC26 liver metastases*

	Treatment	Immunization	Days between tumor implantation and treatment	n	Liver weight (g) + SD	Spleen weight (g) + SD
A	hrR3[a]	None	3	4	1.28 ± 0.15	0.86 ± 0.14
	hrR3 (heat-inactivated)	None	3	4	2.69 ± 0.63[b]	0.83 ± 0.32[c]
	Media	None	3	4	2.35 ± 0.46[b]	0.70 ± 0.07[c]
	*d*120	None	3	4	2.60 ± 1.03[b]	1.02 ± 0.24[c]
	*d*27	None	3	4	3.05 ± 0.46[b]	0.96 ± 0.21[c]
B	hrR3	None	7	5	1.98 ± 0.29	0.96 ± 0.27
	hrR3 (heat-inactivated)	None	7	5	3.07 ± 0.21[d]	0.94 ± 0.31[e]
C	hrR3[f]	KOS	3	7	1.82 ± 0.61	1.03 ± 0.64
	hrR3[g]	Media	3	8	2.10 ± 0.85[h]	0.80 ± 0.41[h]

[a] 1×10^5 MC26 cells in 100 μl HBSS without Ca^{2+} or Mg^{2+} were inoculated into the spleens of BALB/c mice prior to the specified treatment on the specified day. Representative results of three experiments. [b] $P < 0.05$, compared to hrR3 treatment 3 days after tumor implantation. [c] P value not significant, compared to hrR3 treatment 3 days after tumor implantation. [d] $P < 0.05$, compared to hrR3 treatment 7 days after tumor implantation. [e] P value not significant, compared to hrR3 treatment 7 days after tumor implantation. [f] Mice were immunized with 1×10^7 pfu KOS 25 days prior to tumor implantation. [g] Mice were immunized with media 25 days prior to tumor implantation. [h] P value not significant, compared to hrR3 treatment of KOS-immunized mice.

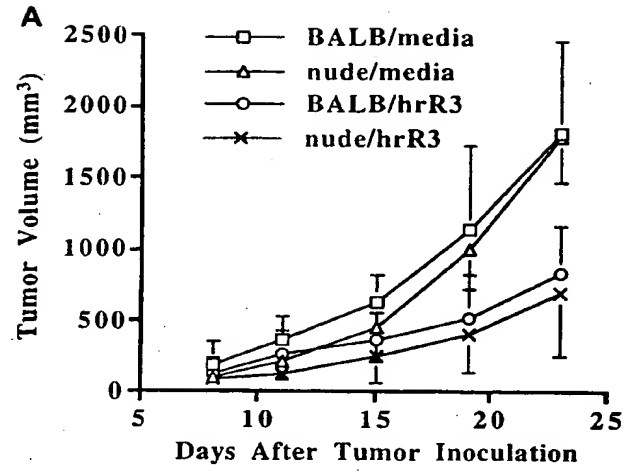
We repeated the same experiment; however, the inoculation of hrR3 or heat-inactivated hrR3 was performed 7 days after tumor cell implantation, at which time tumors were 1–3 mm in size. Significant anti-tumor activity was still observed after a single administration of hrR3 despite the larger initial hepatic tumor burden (Table 1B). These results suggest that hrR3 exerted its anti-tumor effects in the liver after diffuse hepatic metastases had already been established.

Host immune responses do not contribute significantly to hrR3 oncolysis

The striking anti-tumor effects observed as a result of a single intravascular administration of hrR3 could theoretically be a consequence of viral replication and oncolysis or, alternatively, could be a result of anti-tumor immune responses. To examine whether viral replication is required to produce the anti-tumor effects, we established diffuse MC26 liver metastases in another cohort of mice and treated them with an intrasplenic inoculation of either *d*120 or *d*27. Tumor-bearing mice treated with either of these replication-incompetent HSV1 mutants appeared similar to those treated with media or heat-inactivated virus, and many were moribund when killed 14 days after tumor implantation. Livers from these animals appeared identical to those of the mice treated with either media or heat-inactivated virus (Fig. 4*C*). These results strongly suggest that viral replication is required to achieve the anti-tumor effects observed *in vivo*.

We also compared the oncolytic effects of hrR3 against MC26 cells growing in immune-competent, syngeneic BALB/c mice and in immune-incompetent, athymic BALB/c (nu/nu) mice. We chose a flank tumor growth model in order to allow serial tumor size measurements to look for subtle differences in anti-neoplastic efficacy between the two models. Subcutaneous MC26 tumors were established on the flanks of both the immune-competent and immune-incompetent mice, and the tumors were treated with direct intralesional inoculations of either hrR3 or media 8 and 11 days later. The MC26 tumors treated with media grew equally well in both types of mice (**Fig. 5***A*). Intralesional inoculation of hrR3 produced significant anti-tumor effects in both types of mice. These anti-tumor effects were nearly identical, with a 54% and 61% reduction in tumor size 23 days after initial tumor implantation in BALB/c and BALB/c (nu/nu) mice, respectively. These results suggest that the intact immune system in BALB/c mice neither enhances nor attenuates the anti-neoplastic effects of hrR3 infection of MC26 tumors compared with that observed in BALB/c nude mice.

As another measure of the host immune response's role in the observed anti-tumor effects, we examined whether hrR3 treatment of diffuse liver metastases results in a measurable effect against uninfected tumors growing remotely on the flank. We again treated diffuse MC26 liver metastases with a single intrasplenic inoculation of hrR3. Five days later, 2.5×10^6 MC26 cells were implanted s.c. over the flank, and flank tumor size was determined serially. Control mice had MC26 cells implanted into the flank, but did receive hrR3 treatment of liver metastases. None of the flank tumors in either group were rejected and the rate of flank tumor growth was no different in mice treated with hrR3 compared to control mice (Fig. 5*B*). These results again suggest that the principal mechanism by which hrR3 achieves its anti-tumor effects is oncolysis resulting from viral replication rather than induction of host immune responses against the tumor.



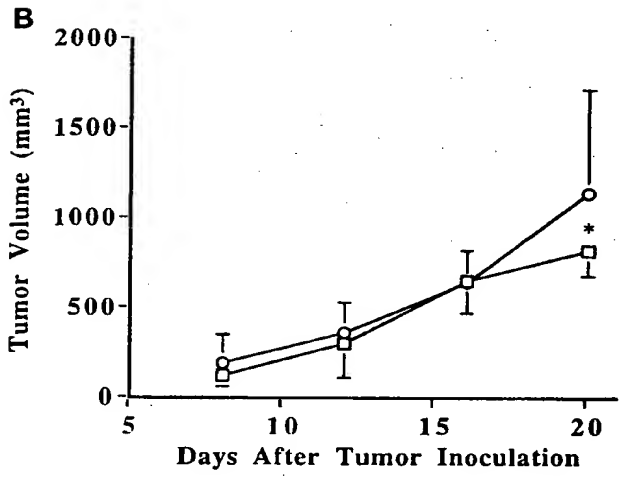


Figure 5. Host immune responses against tumor do not contribute significantly to hrR3 oncolysis. *A)* 2.5×10^7 MC26 cells were inoculated into the flanks of BALB/c mice. Eight and 11 days later, developing tumors were injected with 1×10^8 pfu hrR3 (BALB/hrR3) or media alone (BALB/media). Similary, MC26 cells were inoculated into the flanks of athymic BALB/c mice and subsequently treated with hrR3 (nude/hrR3) or media (nude/media). Flank tumor size was measured every 3–4 days. *B)* MC26 subcutaneous flank tumors were similarly established either in BALB/c mice after treatment of their MC26 liver metastases with an intrasplenic inoculation of hrR3 (squares) or in control mice (circles). Flank tumors were measured twice weekly for calculation of tumor volumes (*difference not statistically significant).

HSV1-mediated oncolysis of diffuse liver metastases is effective in mice immunized against HSV1

The prevalence of preexisting antibodies to HSV1 in some populations in the U.S. is as high as 80% (29), which may reduce the efficacy of HSV1 therapy (30).

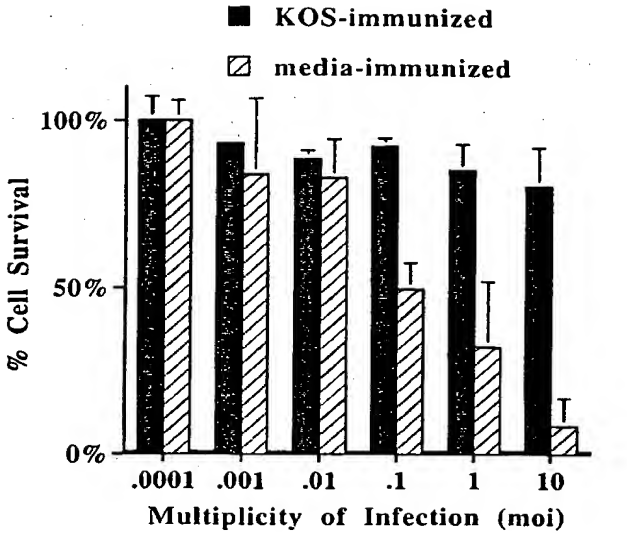


Figure 6. Sera from HSV1-immunized mice neutralize hrR3. hrR3 was incubated with sera collected from BALB/c mice that had been immunized 28 days earlier with KOS (KOS-immunized) or media (media-immunized), and then added to MC26 cells using several moi values. Surviving cells were quantified 6 days later using an MTT assay.

To examine the effect of immunity to HSV1 on hrR3 oncolysis of liver metastases, we vaccinated BALB/c mice with either wild-type HSV1 (KOS strain) or media. The presence of neutralizing antibodies in two mice from each group was confirmed 28 days later (**Fig. 6**). Experimental MC26 liver metastases were established, followed by treatment with hrR3. The presence of neutralizing antibodies in the KOS-vaccinated mice neither enhanced nor reduced the efficacy of hrR3 treatment of the liver metastases (Table 1C).

DISCUSSION

Several biological properties of HSV render it an ideal vector for cancer therapy (reviewed in ref 31). First, tumor cells infected with HSV are destroyed during viral replication; transgene expression is not required for anti-tumor activity (23, 32, 33). Second, these oncolytic effects are present even at extremely low multiplicity of infection (moi) values compared to adenovirus, vaccinia virus, and reovirus (23, 32). Third, HSV's intrinsic thymidine kinase is expressed during viral replication, thereby permitting enhancement of the anti-tumor effect by treatment with prodrugs such as ganciclovir and bromovinyldeoxyuridine (23, 32, 34). Fourth, HSV1 vectors can carry up to 50 kb of transgene sequence, thereby allowing delivery of additional therapeutic genes to increase oncolytic activity (35). And fifth, the virus is

a common pathogen in humans, yet very rarely causes serious medical illness (36).

One of the earliest replication-competent HSV1 mutants examined for its anti-tumor efficacy is *dlsptk*, which is defective in expression of viral thymidine kinase (14). It was proposed that replication of *dlsptk* proceeds selectively in actively dividing cells, which can complement the absence of viral thymidine kinase. Direct inoculation of this vector into intracranial gliomas produces anti-tumor effects in mice (14). The vector that we have investigated in our studies, hrR3, is defective in viral ribonucleotide reductase expression. When inoculated directly into intracranial gliosarcomas, this vector also produces anti-tumor effects (17). Another HSV1 mutant, G207, which is defective in both gamma 1 34.5 and ribonucleotide reductase, has also been inoculated directly into intracranial tumors to produce anti-tumor effects (16). None of these studies have demonstrated that *1)* intravascular delivery of HSV1 can selectively target multiple and diffuse tumors; *2)* HSV1 replication is required to achieve the observed anti-tumor effects; *3)* oncolysis results from viral replication rather than induction of host immune responses against the tumor; *4)* oncolysis is effective in mice immunized against HSV1; and *5)* HSV1-induced oncolysis is an effective therapy for tumors of non-CNS origin. We have provided experimental data that support each of these five important points.

In previous work we demonstrated that hrR3 replicates specifically in liver metastases rather than normal liver, but we did not demonstrate the therapeutic efficacy of this replication (19, 23). In the present study we demonstrate the therapeutic efficacy of hrR3 replication in liver metastases, and have examined the role of host immunity. HSV1 antigens expressed on tumor cells may serve as potent immunogens and modulate the anti-neoplastic activity observed after tumor infection with HSV1. Previous *in vivo* studies examining HSV1 oncolysis have used highly immunogenic tumor models (17), and in some of these models the host immune response contributed significantly to the anti-tumor effect (37, 38). We have used several controls in our experiments and concluded that the host immune response contributed minimally (if at all) to the observed anti-tumor effects in the liver. First, the observed anti-tumor effects on MC26 cells infected with hrR3 were similar in immunocompetent mice and congenitally athymic mice. Second, we could not detect a vaccination response against MC26 tumor cells implanted into the flank after treatment of MC26 liver metastases with hrR3. Third, treatment of liver metastases with replication-incompetent HSV1 mutants *d*120 and *d*27 did not produce any measurable reduction in tumor growth. β-galactosidase is an immunogen expressed by hrR3 but not by *d*120 or d27. However, it is unlikely that this *E. coli* protein accounted for the differences between these viruses in their anti-tumor activity, since it is only one of many immunogens expressed by HSV1.

In an overwhelming majority of patients with solid tumor metastases, most deposits of metastatic tumor are diffuse and not detectable by radiological techniques. This clinical scenario precludes therapeutic approaches that require inoculation of each and every metastatic tumor deposit with an anti-neoplastic agent. Accordingly, we have focused on strategies involving intravascular delivery to target diffuse metastatic deposits. Unlike other solid tumors, colorectal carcinoma commonly spreads to the liver without simultaneous spread to other organs (39). Because of this unique tumor biology, therapies directed specifically against liver metastases have enhanced survival (40).

Much of the safety of oncolytic viral therapy depends on the selectivity of viral replication for tumor cells compared to normal cells. We demonstrate that levels of ribonucleotide reductase are high in colon carcinoma cells and low in hepatocytes and that hrR3 selectively replicates in cells with high levels of ribonucleotide reductase. However, even when administered into the portal vein, hrR3 that passes into the systemic circulation could replicate in actively dividing cell populations outside of the liver. We examined lung and brain sections and did not detect β-galactosidase staining or cytopathic effects (data not shown). It remains to be determined whether hrR3 can be detected in other organs by more sensitive techniques such as polymerase chain reaction, *in situ* hybridization, or immunohistochemical staining for HSV1 proteins.

Although a single administration of hrR3 produced significant anti-tumor activity against macroscopic liver metastases 7 days after implantation, the tumor burden we treated was limited relative to that observed in many clinical scenarios. Most patients with liver metastases with whom this approach can be examined have larger tumor burdens. Because HSV1 replication is several orders of magnitude more robust in human cancer cells than in murine cancer cells, HSV1 oncolytic therapy may be more effective in patients than in mice despite their higher liver tumor burdens. Also, multiple dose administrations may enhance efficacy in patients. Although antibodies to HSV1 are relatively common in humans, our data suggest that this may not adversely affect therapeutic efficacy. Finally, colon carcinoma liver metastases in humans are supplied principally by the hepatic artery, whereas normal liver parenchyma is supplied principally by the portal vein (41). Accordingly, administration of hrR3 into the hepatic artery would be expected to result in higher levels of HSV1 replication in liver metastases compared to adminis-

tration into the portal vein. Though this can be readily accomplished in patients, it is not possible in BALB/c mice.

HSV1 thymidine kinase is expressed during viral replication, which enhances cellular susceptibility to ganciclovir (42). In the present study, we have focused our investigation on the anti-neoplastic activity specifically attributable to HSV1 replication and did not examine the effects of ganciclovir administration. We previously demonstrated that exposure of tumor cells to ganciclovir after administration of hrR3 enhances tumor cell destruction in some cell lines, but not in others (17, 43).

We have demonstrated the therapeutic potential of hrR3 against liver metastases; however, we anticipate that modifications to address three issues will enhance the safety and efficacy of HSV1-based oncolytic therapy. First, ICP6 in hrR3 is inactivated solely by an insertion of the β-galactosidase gene (15). Because expulsion of this inserted gene could reconstitute a wild-type virus, we recently constructed another HSV mutant with a significant portion of ICP6 deleted, which makes it a safer virus to examine in clinical trials (44). Second, hrR3 is most effective against dividing cells; accordingly, G_0 cancer cells may be less susceptible to productive hrR3 infection. Third, actively dividing cell populations in the body other than tumor cells may theoretically be susceptible to hrR3. All three of these issues can be addressed, for example, by construction of an HSV1 vector in which a viral gene required for replication (e.g., ICP4) is regulated by a promoter for a tumor-associated antigen such as carcinoembryonic antigen (CEA). This type of HSV1 vector would not be as susceptible to reconstitution of wild-type virus, could infect nondividing cancer cells, and should not replicate in cells that do not express CEA. Unlike intralesion inoculation, intravascular administration of hrR3 targets widespread disease; therefore, HSV1 mutants engineered to restrict productive infection to tumor cells hold promise as cancer therapeutic agents. FJ

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